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IMMUNOMODULATING COMPOSITIONS, USES THEREFOR AND PROCESSES FOR THEIR PRODUCTION

FIELD OF THE INVENTION

THIS INVENTION relates generally to modulation of immune responses. More particularly, the present invention relates to the use of at least one set of peptides in compositions and methods for modulating an immune response to one or more polypeptide antigens, wherein the sequences of a respective set of peptides are derived in whole or in part from a single polypeptide antigen. Individual peptides of a respective peptide set comprise different portions of an amino acid sequence corresponding to a single polypeptide antigen and display partial sequence identity or similarity to at least one other peptide of the same set of peptides. The invention also extends to methods of using such peptides in a range of preventive, diagnostic and therapeutic applications.

Bibliographic details of various publications numerically referred to in this specification are collected at the end of the description.

BACKGROUND OF THE INVENTION

Since its discovery almost 20 years ago, the human immunodeficiency virus type-1 (HIV-1) has claimed more than 22 million lives and is continuing to devastate communities worldwide (1). Forty-two million people are currently living with HIV-1 and, despite efforts to modify high-risk behaviour, an estimated 5 million new infections occurs yearly (2). A safe and effective preventative or therapeutic vaccine is desperately needed.

The elimination of persistent intracellular pathogens such as replicating viruses requires the mobilisation of cell-mediated immunity (CMI). CD8⁺ cytotoxic T lymphocytes (CTL) are the primary effector cells of CMI; they kill virally-infected cells by recognising viral peptides presented on the cell surface in the context of MHC class I molecules. Prior to the appearance of virus-specific antibodies, a robust HIV-1-specific CTL response temporally correlates with reduced viremia during the acute stage of HIV-1 infection (3, 4). Furthermore, strong CTL responses are associated with reduced HIV-1 viremia during chronic infection (5, 6), whereas a decline in HIV-1-specific CTL is linked to rapid progression to AIDS (4, 7-9).

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Early HIV-1 vaccine strategies were based on whole inactivated virus and recombinant structural proteins such as the envelope (env) glycoprotein. Non-human primate models revealed only limited strain-specific protection by these vaccines against pathogenic simian immunodeficiency virus (SIV) and highly pathogenic SHIV (SIV-HIV-1 chimeric) challenges (10-13). The first human phase III trials also failed to show efficacy (14).

Particle- and recombinant whole protein-based vaccines, although safe, favour the generation of antibodies that are of insufficient protection. Alternatively, intracellularly expressed antigens are subsequently more likely to induce CTL responses. Live-attenuated viruses generate potent cell-mediated immunity (CMI) responses, however their clinical safety is of concern (15). Consequently, much focus has shifted toward genetically engineered vectors (such as DNA plasmids and poxviruses) expressing HIV-1/SIV genes (such as *env*, *gag* and *pol*) (16).

It is not known which immune-target antigens are protective, but a large breadth of T cell responses has been shown to reduce the opportunity for escape mutants to arise (17). It is this large breadth of potential epitopes, however, which renders the construct of large vectors frequently difficult and as well as potential safety issues. Concerns have been raised about the potential ability of DNA vaccines to integrate with host DNA, as well as the safety of viral vector vaccines in immunocompromised hosts. These represent the significant regulatory hurdles for these recombinant vaccines.

Also, despite significant advances towards understanding how T and linear B cell epitopes are processed and presented to the immune system, the full potential of epitope-based vaccines has not been fully exploited. The main reason for this is the large number of different T cell epitopes, which must be identified for inclusion into such vaccines to cover the extreme human leucocyte antigen (HLA) polymorphism in the human population.

Infusion of whole antigen-pulsed or single epitope-pulsed antigen presenting cells (APC) has previously been reported to be immunogenic in murine models (22-27). However, other reports in inbred mouse models suggest the infusion of cells pulsed with single peptides may even be tolerogenic (induces a state of tolerance to the antigen which would be counterproductive for a vaccine) (28-31).

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SUMMARY OF THE INVENTION

The present invention discloses the discovery that autologous cells, which have been contacted with overlapping peptides spanning a viral polypeptide antigen of interest produce a strong immunogenic response in an outbred population that protects against subsequent viral challenge. The present inventors propose that similar protective responses would be achieved using systemic administration of the overlapping peptides *per se*. The use of multiple overlapping peptides provides several advantages, including reducing the emergence of escape mutants and the facile production of peptide-based immunogenic compositions without prior knowledge of any epitopes. In this regard, the sequence overlap between peptides reduces or prevents loss of potential epitopes, which broadens the immunological coverage of the composition to cover potentially the diversity in the major histocompatibility complex across an outbred population.

Accordingly, in one aspect of the present invention, there is provided at least one set of peptides for modulating an immune response to one or more polypeptides of interest. Individual peptides of a respective set comprise different portions of an amino acid sequence corresponding to a single polypeptide of interest and display partial sequence identity or similarity to at least one other peptide of the same set of peptides. In certain embodiments, at least 2, 3, 4, 5, 6, 7 sets of peptides are employed, wherein peptide sequences for each set are derived from a distinct polypeptide of interest.

The partial sequence identity or similarity is typically contained at one or both ends of an individual peptide. Suitably, at one or both of these ends there are at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 contiguous amino acid residues whose sequence is identical or similar to an amino acid sequence contained within at least one other of the peptides.

In certain embodiments, the peptide is at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30 amino acid residues in length and suitably no more than about 500, 200, 100, 80, 60, 50, 40 amino acid residues in length.

In certain embodiments, the peptide sequences are derived from at least about 30, 40, 50, 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% of the sequence corresponding to the polypeptide of interest.

The polypeptide of interest is suitably an antigen selected from a protein antigen, a particulate antigen, an alloantigen, an autoantigen or an allergen, or an immune complex. In certain embodiments, the polypeptide of interest is a disease- or condition-associated

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polypeptide such as but not limited to a polypeptide produced by a pathogenic organism or a cancer. Examples of pathogenic organisms include, but are not restricted to, yeast, viruses, bacteria, helminths, protozoans and mycoplasmas. Examples of cancers include, but are not restricted to, melanoma, lung cancer, breast cancer, cervical cancer, prostate cancer, colon cancer, pancreatic cancer, stomach cancer, bladder cancer, kidney cancer, post transplant lymphoproliferative disease (PTLD), Hodgkin's Lymphoma and the like.

In another aspect, the invention provides antigen-presenting cells or their precursors which have been contacted with a set of peptides as broadly described above for a time and under conditions sufficient for the peptides or processed forms thereof to be presented by the antigen-presenting cells or by their precursors.

In a related aspect, the invention provides a process for producing antigen-presenting cells for modulating an immune response to a polypeptide of interest. The process generally comprises contacting antigen-presenting cells or their precursors with at least one set of peptides as broadly described above for a time and under conditions sufficient for the peptides or processed form thereof to be presented by the antigen-presenting cells or by their precursors. Suitably, when precursors are used, the precursors are cultured for a time and under conditions sufficient to differentiate antigen-presenting cells from the precursors.

In one embodiment, the or each set of peptides is contacted with substantially purified antigen-presenting cells or their precursors. In another embodiment, the or each set of peptides is contacted with a heterogeneous population of antigen-presenting cells or their precursors. In this embodiment, the heterogeneous pool of cells can be blood or peripheral blood mononuclear cells. Typically, the antigen-presenting cells or their precursors are selected from monocytes, macrophages, cells of myeloid lineage, B cells, dendritic cells or Langerhans cells.

The antigen-presenting cells broadly described above are also useful for producing lymphocytes, including T lymphocytes and B lymphocytes, for modulating an immune response to a specified antigen or group of antigens. Accordingly, in yet another aspect, the invention provides a method for producing antigen-specific lymphocytes. The method comprises contacting a population of lymphocytes, or their precursors, with an antigen-presenting cell as broadly described above for a time and under conditions

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sufficient to produce the antigen-specific lymphocytes that modulate an immune response to at least one polypeptide from which the overlapping peptides were derived.

5 In yet another aspect, the invention contemplates a composition comprising at least one set of peptides, or the antigen-presenting cells, or the lymphocytes, as broadly described above, and a pharmaceutically acceptable carrier and/or diluent. In certain embodiments, the composition may further comprise an adjuvant.

10 In yet another aspect, the invention embraces a method for modulating an immune response to a polypeptide of interest, comprising administering to a patient in need of such treatment at least one set of peptides, or the antigen-presenting cells, or the lymphocytes, or the composition as broadly described above for a time and under conditions sufficient to modulate the immune response.

15 In a related aspect, the invention encompasses a method for treatment and/or prophylaxis of a disease or condition associated with the presence of a polypeptide of interest, comprising administering to a patient in need of such treatment or prophylaxis an effective amount of at least one set of peptides, or the antigen-presenting cells, or the lymphocytes, or the composition as broadly described above. In some embodiments, peptides or antigen-presenting cells or the lymphocytes are administered systemically, typically by injection.

20 In still yet another aspect, the invention contemplates the use of at least one set of peptides, or of the antigen-presenting cells, or of the lymphocytes, as broadly described above, in the preparation of a medicament for modulating an immune response to a polypeptide of interest or for treating or preventing a disease or condition associated with the presence of a polypeptide of interest.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation showing one embodiment of an SIV_{mac236} gag peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 125 is 14 amino acids in length. The full-length gag sequence is modified from the HIV sequence database <http://hiv-web.lanl.gov>.

Figure 2 is a diagrammatic representation showing one embodiment of an SIV_{mac236} pol peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. The full-length pol sequence is modified from the HIV sequence database <http://hiv-web.lanl.gov>.

Figure 3 is a diagrammatic representation showing one embodiment of an SIV_{mac236} nef peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. The full-length nef sequence is modified from the HIV sequence database <http://hiv-web.lanl.gov>.

Figure 4 is a diagrammatic representation showing one embodiment of an SHIV_{SF162P3} env peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 211 is 14 amino acids in length. *Peptide overlaps preceding peptide by 10 amino acids to eliminate a forbidden Q n-terminal peptide. The full-length env sequence is modified from the HIV sequence database <http://hiv-web.lanl.gov>.

Figure 5 is a schematic representation of an *in vivo* CTL killing assay performed at weeks 10, 15 and 20.

Figure 6 is a graphical representation showing *in vivo* CTL killing of SIVgag overlapping peptide-pulsed cells. Two weeks after the FPV-boost (week 10), 3 equal PBMC populations were labelled with SNARF (2.5 μ M) or CFSE (2.5 μ M or 0.25 μ M) and were pulsed with SIVpol, nef or gag overlapping peptide pools (OPAL), respectively. Blood sampled at 5 min, and at 4 and 16 h post-OPAL infusion was RBC-lysed and 10⁶ lymphocyte events were acquired by flow cytometry. At 5 min, all 3 populations of

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labelled PBMC are of relatively equal numbers. By 4 and 16 hours, 2xDNA/FPV-immunised monkey H20 displayed 27.3% and 76.0% clearance of SIVgag-pulsed PBMC with respect to SIVnef-pulsed PBMC, respectively, whereas no SIVgag-specific killing was observed in control-immunised monkey E20. Note that less events were collected at 4 h than 16 h.

Figure 7 is a graphical representation showing vigorous killing of SIVgag- and SIVpol-pulsed PBMC following SHIV challenge. Two weeks after SHIV challenge (week 20), equal PBMC populations were labelled with SNARF (5 μ M) or CFSE (6 μ M or 2.5 μ M) and were pulsed with SIVpol, no peptide, or SIVgag overlapping peptide pools (OPAL), respectively. 10⁶ RBC-lysed lymphocyte events were acquired by flow cytometry. 2xDNA/FPV-immunised monkeys H20 and H21, Displayed 92.3% and 98.3% killing of SIVgag-pulsed PBMC. These animals received 2 separate infusions of SIVpol- pulsed PBMC, furthermore displaying >99% SIVpol-specific killing. Previously CFSE-labelled PBMC were accounted for by flow cytometric analysis of 10⁶ lymphocytes immediately prior to OPAL-infusion (not shown).

Figure 8 is a photographic representation showing a boost in T-cell immunogenicity 1 week following OPAL-infusion analysed by IFN γ ELISpot. A boost in SIVgag and pol peptide pool responses is evident in 2xDNA/FPV-immunised monkey H21, where as a primed response to SIVpol peptide pool is detected in control-immunised monkey E20 (week 10 shown above).

Figure 9 is a graphical representation depicting IFN γ ELISpot analysis 1 week following OPAL infusion at week 10. A boost in T-cell immunogenicity to SIVgag, pol and nef overlapping peptide pools by OPAL infusion at week 10 was analysed 1 week later by ELISpot. Increased responses to SIVgag were detected in all four 2xDNA/FPV-immunised animals. Increased SIVpol responses were present in the 2xDNA/FPV-immunised monkeys, H20 and H21 (monkeys B00 and H8 did not receive any pol-pulsed PBMC), and in one control-immunised monkey, E20. No responses to SIVnef were primed in any animals. *IFN γ spots in monkeys E20 (prior to OPAL infusion) and B00 (post-OPAL infusion) were excluded due to ELISpot developmental problems.

Figure 10 is a graphical representation showing IFN γ ELISpot analysis 1 week following OPAL infusion at week 15. A boost in T-cell immunogenicity to SIVgag, pol, nef and HIV-1env overlapping peptide pools by OPAL infusion at week 15 was analysed 1

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week later by INF γ ELISpot. Increased responses to SIVgag were detected in all four 2xDNA/FPV-immunised animals. SIVpol responses were marginally increased (or primed) in monkeys, E22, B00, H20 and H21. Increased responses to WI SIV were evident in all animals, whereas no responses were detected for SIVnef or HIV-env in any animals.

5 Figure 11 is a graphical representation depicting mean INF γ ELISpot of immunogenicity of OPAL infusion. Mean INF γ ELISpot responses to (A) SIVgag and (B) SIVpol overlapping peptide pool of control- and 2xDNA/FPV-immunised animals receiving OPAL infusions (bold) were compared to animals receiving equivalent immunisations but no OPAL infusions, before an after the OPAL infusions given at weeks
10 10 and 15 following the immunisation. For the comparison of SIVpol-specific responses, 2xDNA/FPV-immunised animals were grouped based on receiving either 1 (B00 and H8) or 2 (H20 and H21) doses of pol-OPAL infusions.

15 Figure 12 is a graphical representation showing the outcome of SHIV intrarectal challenge. At week 18 all control-and 2xDNA/FPV-immunised macaques were challenged intrarectally with SHIV_{mn229} and were assessed for plasma SHIV RNA viral load and CD4+ T cell count over the course of the infection. Recipients of OPAL infusion were compared to their respective immunised non-OPAL recipients. Group comparisons indicate mean \pm SE. 2xDNA/FPV-immunised macaques receiving OPAL infusions were further grouped based on receiving either 1 or 2 separate doses of pol-pulsed PBMC (B00
20 & H8, and H20 & H21, respectively).

25 Figure 13 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Gag peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 124 is 12 amino acids in length.
The full-length Gag sequence is modified from the HIV sequence database.

30 Figure 14 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Nef peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 49 is 14 amino acids in length.
The full-length Nef sequence is modified from the HIV sequence database.

Figure 15 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Pol peptide pool sequence. Each peptide is 15 amino acids in length and

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overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 248 is 14 amino acids in length. The full-length Pol sequence is modified from the HIV sequence database HIV-1.

5 Figure 16 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Rev peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 27 is 13 amino acids in length. The full-length Rev sequence is modified from the HIV sequence database.

10 Figure 17 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Tat peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 24 is 14 amino acids in length. The full-length Tat sequence is modified from the HIV sequence database.

15 Figure 18 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Vif peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 46 is 12 amino acids in length. The full-length Vif sequence is modified from the HIV sequence database.

20 Figure 19 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Vpr peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 22 is 12 amino acids in length. The full-length Vpr sequence is modified from the HIV sequence database.

25 Figure 20 is a diagrammatic representation showing one embodiment of an HIV-1 consensus B clade Vpu peptide pool sequence. Each peptide is 15 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide 18 is 13 amino acids in length. The full-length Vpu sequence is modified from the HIV sequence database.

30 Figure 21 is a diagrammatic representation showing one embodiment of a peptide pool sequence of HCV 1a H77. Each peptide is 18 amino acids in length and overlaps the preceding peptide by 11 amino acids. Peptides are numbered periodically, indicated in brackets left of the peptide sequence. Peptide couples 25 & 26, 153 & 154, 220 & 221, 239

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& 240, 242 & 243, 244 & 245, 345 & 346 are divided into 15- and 14-mers due to problematic sequences of the original 18-mer peptide. The full-length HCV 1a H77 sequence is modified from the HCV sequence database

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

The articles "*a*" and "*an*" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "*an element*" means one element or more than one element.

The term "*about*" is used herein to refer to conditions (*e.g.*, amounts, concentrations, time etc) that vary by as much as 30%, preferably by as much as 20%, and more preferably by as much as 10% to a specified condition.

By "*antigen*" is meant all, or part of, a protein, peptide, or other molecule or macromolecule capable of eliciting an immune response in a vertebrate animal, preferably a mammal. Such antigens are also reactive with antibodies from animals immunised with said protein, peptide, or other molecule or macromolecule.

By "*antigen-binding molecule*" is meant a molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins, immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity.

By "*autologous*" is meant something (*e.g.*, cells, tissues etc) derived from the same organism.

The term "*allogeneic*" as used herein refers to cells, tissues, organisms etc that are of different genetic constitution.

Throughout this specification, unless the context requires otherwise, the words "*comprise*", "*comprises*" and "*comprising*" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

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By "*corresponds to*" or "*corresponding to*" is meant a polynucleotide (a) having a nucleotide sequence that is substantially identical or complementary to all or a portion of a reference polynucleotide sequence or (b) encoding an amino acid sequence identical to an amino acid sequence in a peptide or protein. This phrase also includes within its scope a
5 peptide or polypeptide having an amino acid sequence that is substantially identical or similar to a sequence of amino acids in a reference peptide or protein.

As used herein, the terms "*culturing*", "*culture*" and the like refer to the set of procedures used *in vitro* where a population of cells (or a single cell) is incubated under conditions which have been shown to support the growth or maintenance of the cells *in*
10 *vitro*. The art recognises a wide number of formats, media, temperature ranges, gas concentrations etc. which need to be defined in a culture system. The parameters will vary based on the format selected and the specific needs of the individual who practices the methods herein disclosed. However, it is recognised that the determination of culture parameters is routine in nature.

15 By "*effective amount*", in the context of modulating an immune response or treating or preventing a disease or condition, is meant the administration of that amount of composition to an individual in need thereof, either in a single dose or as part of a series, that is effective for that modulation, treatment or prevention. The effective amount will vary depending upon the health and physical condition of the individual to be treated, the
20 taxonomic group of individual to be treated, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

By "*expression vector*" is meant any autonomous genetic element capable of directing the synthesis of a protein encoded by the vector. Such expression vectors are
25 known by practitioners in the art.

The term "*gene*" as used herein refers to any and all discrete coding regions of the cell's genome, as well as associated non-coding and regulatory regions. The gene is also intended to mean the open reading frame encoding specific polypeptides, introns, and adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of
30 expression. In this regard, the gene may further comprise control signals such as promoters, enhancers, termination and/or polyadenylation signals that are naturally associated with a given gene, or heterologous control signals. The DNA sequences may be

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cDNA or genomic DNA or a fragment thereof. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

Reference herein to "*immuno-interactive*" includes reference to any interaction, reaction, or other form of association between molecules and in particular where one of the
5 molecules is, or mimics, a component of the immune system.

By "*isolated*" is meant material that is substantially or essentially free from components that normally accompany it in its native state.

By "*modulating*" is meant increasing or decreasing, either directly or indirectly, the immune response of an individual.

10 The term "*operably connected*" or "*operably linked*" as used herein means placing a structural gene under the regulatory control of a promoter, which then controls the transcription and optionally translation of the gene. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the genetic
15 sequence or promoter at a distance from the gene transcription start site that is approximately the same as the distance between that genetic sequence or promoter and the gene it controls in its natural setting; *i.e.* the gene from which the genetic sequence or promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, the preferred positioning of a regulatory
20 sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting; *i.e.* the genes from which it is derived.

The term "*patient*" refers to patients of mammalian, especially human, or other animal origin and includes any individual it is desired to examine or treat using the methods of the invention. However, it will be understood that "*patient*" does not imply that
25 symptoms are present. Suitable animals that fall within the scope of the invention include, but are not restricted to, primates, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes, reptiles, avians, fish).

30 By "*pharmaceutically-acceptable carrier*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in topical or systemic administration.

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The term "*polynucleotide*" or "*nucleic acid*" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to oligonucleotides greater than 30 nucleotides in length.

5 "*Polypeptide*", "*peptide*" and "*protein*" are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers.

10 Reference herein to a "*promoter*" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (*i.e.* upstream activating sequences, enhancers and silencers) which alter gene expression in response to
15 developmental and/or environmental stimuli, or in a tissue-specific or cell-type-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. Preferred promoters according to the invention may contain additional copies of
20 one or more specific regulatory elements to further enhance expression in a cell, and/or to alter the timing of expression of a structural gene to which it is operably connected.

 The term "*purified peptide*" means that the peptide is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the peptide is derived, or substantially free from chemical precursors or other chemicals when
25 chemically synthesised. "Substantially free" means that a preparation of a peptide of the invention is at least 10% pure. In certain embodiments, the preparation of peptide has less than about 30%, 25%, 20%, 15%, 10% and desirably 5% (by dry weight), of non-peptide protein (also referred to herein as a "contaminating protein"), or of chemical precursors or non-peptide chemicals. The invention includes isolated or purified preparations of at least
30 0.01, 0.1, 1.0, and 10 milligrams in dry weight.

 The term "*recombinant polynucleotide*" as used herein refers to a polynucleotide formed *in vitro* by the manipulation of nucleic acid into a form not normally found in

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nature. For example, the recombinant polynucleotide may be in the form of an expression vector. Generally, such expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleotide sequence.

By "*recombinant polypeptide*" is meant a polypeptide made using recombinant techniques, *i.e.*, through the expression of a recombinant polynucleotide.

By "*reporter molecule*" as used in the present specification is meant a molecule that, by its chemical nature, provides an analytically identifiable signal that allows the detection of a complex comprising an antigen-binding molecule and its target antigen. The term "*reporter molecule*" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

The term "*sequence identity*" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "*percentage of sequence identity*" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, I) or the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "*sequence identity*" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software.

"*Similarity*" refers to the percentage number of amino acids that are identical or constitute conservative substitutions as defined in Table B *infra*. Similarity may be determined using sequence comparison programs such as GAP (Deveraux *et al.* 1984, *Nucleic Acids Research* 12, 387-395). In this way, sequences of a similar or substantially different length to those cited herein might be compared by insertion of gaps into the alignment, such gaps being determined, for example, by the comparison algorithm used by GAP.

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Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (*i.e.*, only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, *Nucl. Acids Res.* 25:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998, Chapter 15.

By "*substantially purified population*" and the like is meant that greater than about 80%, usually greater than about 90%, more usually greater than about 95%, typically greater than about 98%, and more typically greater than about 99% of the cells in the population are antigen-presenting cells of a chosen type.

By "*vector*" is meant a nucleic acid molecule, preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, or plant virus, into which a nucleic acid sequence may be inserted or cloned. A vector preferably contains one or more unique

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restriction sites and may be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an
5 extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a linear or closed circular plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s)
10 into which it has been integrated. A vector system may comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may also include a selection marker such as an antibiotic
15 resistance gene that can be used for selection of suitable transformants.

2. *Immunomodulating sets of overlapping peptides*

The present invention is predicated in part on the discovery that antigen-presenting cells contacted *ex vivo* with a set of overlapping peptides spanning a viral polypeptide antigen of interest (also referred to herein as **Overlapping Peptide-pulsed**
20 **Autologous cells**, OPAL) are effective in producing a strong immunogenic response that protects against subsequent viral challenge in an outbred population, without prior knowledge of the epitopes of the antigen. Since antigen-presenting cells form a significant part of the circulatory system, it is proposed that systemic delivery of the overlapping peptides *per se* will produce a similar protective effect. Accordingly, the present invention
25 broadly provides a set of peptides for modulating an immune response to a polypeptide of interest, wherein individual peptides comprise different portions of an amino acid sequence corresponding to the polypeptide of interest and display partial sequence identity or similarity to at least one other peptide of the set.

The partial sequence identity or similarity is typically contained at one or both
30 ends of an individual peptide. In one embodiment, there are at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50 contiguous amino acid residues at one or both ends of an individual peptide, whose sequence is identical or similar to an amino acid sequence

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contained within at least one other of the peptides. In an alternate embodiment, there are less than 500, 100, 50, 40, 30 contiguous amino acid residues at one or both ends of an individual peptide, whose sequence is identical or similar to an amino acid sequence contained within at least one other of the peptides. Such 'sequence overlap' is advantageous to prevent or otherwise reduce the loss of any potential epitopes contained within a polypeptide of interest. One example, the sequence overlap is 11 amino acid residues.

Typically, when peptides have partial sequence similarity, their sequences will usually differ by one or more conserved and/or non-conserved amino acid substitutions. Exemplary conservative substitutions are listed in the following table.

TABLE A

| <i>Original Residue</i> | <i>Exemplary Substitutions</i> | <i>Original Residue</i> | <i>Exemplary Substitutions</i> |
|-------------------------|--------------------------------|-------------------------|--------------------------------|
| Ala | Ser | Leu | Ile, Val |
| Arg | Lys | Lys | Arg, Gln, Glu |
| Asn | Gln, His | Met | Leu, Ile, |
| Asp | Glu | Phe | Met, Leu, Tyr |
| Cys | Ser | Ser | Thr |
| Gln | Asn | Thr | Ser |
| Glu | Asp | Trp | Tyr |
| Gly | Pro | Tyr | Trp, Phe |
| His | Asn, Gln | Val | Ile, Leu |
| Ile | Leu, Val | | |

Conserved or non-conserved substitutions may correspond to polymorphisms in a polypeptide of interest. Polymorphic polypeptides are expressed by various pathogenic organisms and cancers. For example, the polymorphic polypeptides may be expressed by different viral strains or clades or by cancers in different individuals.

The peptides of the invention may be of any suitable size that can be utilised to elicit an immune response to a polypeptide of interest. A number of factors can influence

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the choice of peptide size. For example, the size of a peptide can be chosen such that it includes, or corresponds to the size of, T cell epitopes and/or B cell epitopes, and their processing requirements. Practitioners in the art will recognise that class I-restricted T cell epitopes are typically between 8 and 10 amino acid residues in length and if placed next to
5 unnatural flanking residues, such epitopes can generally require 2 to 3 natural flanking amino acid residues to ensure that they are efficiently processed and presented. Class II-restricted T cell epitopes usually range between 12 and 25 amino acid residues in length and may not require natural flanking residues for efficient proteolytic processing although it is believed that natural flanking residues may play a role. Another important feature of
10 class II-restricted epitopes is that they generally contain a core of 9-10 amino acid residues in the middle which bind specifically to class II MHC molecules with flanking sequences either side of this core stabilising binding by associating with conserved structures on either side of class II MHC antigens in a sequence independent manner. Thus the functional region of class II-restricted epitopes is typically less than about 15 amino acid
15 residues long. The size of linear B cell epitopes and the factors effecting their processing, like class II-restricted epitopes, are quite variable although such epitopes are frequently smaller in size than 15 amino acid residues. From the foregoing, it is advantageous, but not essential, that the size of the peptide is at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30 amino acid residues. Suitably, the size of the peptide is no more than about 500, 200, 100,
20 80, 60, 50, 40 amino acid residues. In one embodiment, the size of the peptide is large enough to minimise loss of T cell and/or B cell epitopes. In another embodiment, the size of the peptide is sufficient for presentation by an antigen-presenting cell of a T cell and/or a B cell epitope contained within the peptide. In one example of this embodiment, the size of the peptide is about 15 amino acid residues.

25 The polypeptide of interest is suitably a disease- or condition-associated antigen. In one embodiment, the disease- or condition-associated antigen is produced by a pathogenic organism. Pathogenic organisms include, but are not restricted to, yeast, viruses, bacteria, helminths, protozoans and mycoplasmas. Natural hosts for such organisms may be selected from mammals, avians and fish. In one embodiment, the
30 pathogenic organism is a virus. Suitable viruses include RNA viruses or DNA viruses. For example, viruses may be selected from human immunodeficiency virus (HIV), herpes virus, cytomegalovirus, rabies virus, influenza virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, Sendai virus, Rous sarcoma virus, feline leukemia virus, Reo virus, polio

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virus, human serum parvo-like virus, simian virus 40, respiratory syncytial virus, mouse mammary tumour virus, Varicella-Zoster virus, Dengue virus, rubella virus, measles virus, adenovirus, human T-cell leukemia viruses, Epstein-Barr virus, cytomegalovirus, flavivirus such as Japanese encephalitis virus, arboviruses such as bunyaviruses, reoviruses, 5 rhabdoviruses and togaviruses, murine leukemia virus, mumps virus, vesicular stomatitis virus, Sindbis virus, lymphocytic choriomeningitis virus, wart virus, blue tongue virus, Newcastle Disease virus, Infectious Bursal Disease virus, fowl pox virus, sheep pox virus, Laryngotracheitis virus, Infectious Bronchitis of poultry virus, Gumboro Winterfield virus, Infectious Bronchitis and Rinderpest virus. In one example of the present invention, the 10 virus is HIV and the polypeptide of interest is suitably selected from env, gag, pol, vif, vpr, tat, rev, vpu and nef, or combination thereof.

In another embodiment, the pathogenic organism is a bacterium, which includes, but is not restricted to, *Escherichia* species (e.g., *Escherichia coli*), *Neisseria* species (e.g., *Neisseria gonorrhoeae*, *Neisseria meningitidis*), *Meningococcal* species (e.g.,), 15 *Haemophilus* species (e.g., *Haemophilus influenzae* B), *Pneumococcus* species, *Salmonella* species (e.g.,), *Streptococcal* species (e.g., *Streptococcus pyogenes*, *Streptococcus agalactiae*), *Legionella* species (e.g., *Legionella pneumophila*), *Pseudomonas* species (e.g., *Pseudomonas aeruginosa*) and *Mycobacterium* species (e.g., *Mycobacterium leprae*, *Mycobacterium tuberculosis*), *Treponema* species (e.g., *Treponema pallidum*), Lyme 20 disease spirochetes, *Brucella* species (e.g., *Brucella abortus*), and Tetanus toxin.

In yet another embodiment, the pathogenic organism is a protozoan which is suitably selected from the group consisting of *Plasmodium* species (e.g., *Plasmodium falciparum*, *Plasmodium vivax*), *Toxoplasma* species (e.g., *Toxoplasma gondii*), *Trypanosoma* species (e.g., *Trypanosoma rangeli*, *Trypanosoma cruzi*, *Trypanosoma 25 rhodesiense*, *Trypanosoma brucei*), *Schistosoma* species (e.g., *Schistosoma mansoni*, *Schistosoma japonicum*), *Giardia* species, *Babesia* species (e.g., *Babesia bovis*), *Elmeria tenella*, *Onchocerca volvulus*, *Leishmania* species (e.g., *Leishmania tropica*), *Trichinella spiralis*, *Onchocerca volvulus*, *Theileria parva*, *Taenia hydatigena*, *Taenia ovis*, *Taenia saginata*, *Echinococcus granulosus* and *Mesocystoides corti*.

30 In still another embodiment, the pathogenic organism is a mycoplasma which includes, but is not limited to, *Mycoplasma arthritidis*, *Mycoplasma hyorhinis*, *Mycoplasma orale*, *Mycoplasma arginini*, *Acholeplasma laidlawii*, *Mycoplasma salivarum*, and *Mycoplasma pneumoniae*.

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Alternatively, the disease- or condition-associated antigen is an antigen expressed by a cancer or tumour, which is suitably selected from melanoma, lung cancer, breast cancer, cervical cancer, prostate cancer, colon cancer, pancreatic cancer, stomach cancer, bladder cancer, kidney cancer, post transplant lymphoproliferative disease (PTLD), Hodgkin's Lymphoma and the like. In one embodiment, the cancer or tumour relates to melanoma. In this embodiment, the polypeptide of interest can be a melanocyte differentiation antigen which is suitably selected from gp100, MART, TRP-1, Tyros, TRP2, MC1R, MUC1F, MUC1R or a combination thereof. Alternatively, the polypeptide of interest is a melanoma-specific antigen which is suitably selected from BAGE, GAGE-1, gp100In4, MAGE-1, MAGE-3, PRAME, TRP2IN2, NYNSO1a, NYNSO1b, LAGE1 or a combination thereof.

In one example of the present invention, the size of individual peptides is about 14 or 15 amino acid residues and the sequence overlap at one or both ends of an individual peptide is about 11 amino acid residues. However, it will be understood that other suitable peptide sizes and sequence overlap sizes are contemplated by the present invention, which can be readily ascertained by persons of skill in the art.

It is advantageous but not necessary to utilise the entire sequence of a polypeptide of interest for producing a set of overlapping peptides. Typically, at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the sequence corresponding to a polypeptide of interest is used to produce the overlapping peptides of the invention. However, it will be understood that the more sequence information from a polypeptide of interest that is utilised to produce the overlapping peptides, the greater the population coverage will be of the overlapping peptides as an immunogen. Suitably, no sequence information from the polypeptide of interest is excluded (e.g., because of an apparent lack of immunological epitopes). If required, variable and/or hypervariable sequences within a polypeptide of interest can be included in the construction of an overlapping set of peptides. In this embodiment, multiple peptides are synthesised to cover the variation in amino acid residue at individual positions of a polypeptide of interest. Peptide sequences may include additional sequences that are not derived from a polypeptide of interest. These additional sequences may have various functions, including improving solubility or immunogenicity or facilitating purification. Typically, such additional sequences are contained at one or both ends of a respective peptide.

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Persons of skill in the art will appreciate that when preparing a set of overlapping peptides according to the invention, it may be advantageous to use sequence information from a plurality of different polypeptides produced by a pathogenic organism or expressed in a cancer. Accordingly, in certain embodiments, at least 2, 3, 4, 5, 6, 7, 9, 10, 15, 20 other sets of peptides are used for the production of the immunomodulating compositions of the invention, wherein the sequences of a respective other set of peptides are derived from a distinct polypeptide of interest and wherein individual peptides of the respective other set display partial sequence identity or similarity to at least one other peptide of a corresponding set of peptides. It is advantageous in this respect to utilise as many polypeptides as possible from, or in relation to, a particular source in the construction of sets of overlapping peptides. Suitably, at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and desirably 100%, of the polypeptides expressed by the source is used in the construction of the corresponding sets of overlapping peptides. Exemplary viral polypeptides that can be used for such construction include, but are not restricted to, latent polypeptides, regulatory polypeptides or polypeptides expressed early during their replication cycle. Suitably, polypeptides from a protozoan, bacterium, mycoplasma, fungus or helminth include, but are not restricted to, secretory polypeptides, regulatory polypeptides and polypeptides expressed on the surface of these organisms. Polypeptides from a cancer or tumour, which can be used for the construction of overlapping peptide sets, are suitably cancer-specific polypeptides.

Representative overlapping peptide sets for modulating the immune response to simian immunodeficiency virus (SIV) and/or the chimeric SIV-HIV-1 (SHIV) can be based on one or more polypeptides selected from SIV gag, pol, nef or SHIV env as for example presented in Figures 1 to 4. Exemplary overlapping peptide sets for modulating the immune response to HIV-1 can be based on one or more polypeptides selected from HIV Gag, Nef, Pol, Rev, Tat, Vif, Vpr and Vpu as for example set forth in Figures 13 to 20. Exemplary overlapping peptide sets for modulating the immune response to HCV 1a can be based on the HCV 1a H77 polyprotein sequence as for example set forth in Figure 21.

The overlapping peptide sets of the invention may be prepared by any suitable procedure known to those of skill in the art. For example, the peptide sets can be synthesised conveniently using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 of Atherton and Shephard (1989, Solid Phase Peptide Synthesis: A

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Practical Approach. IRL Press, Oxford) and in Roberge *et al* (1995, *Science* 269: 202). Syntheses may employ, for example, either *t*-butyloxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc) chemistries (see Chapter 9.1, of Coligan *et al.*, CURRENT PROTOCOLS IN PROTEIN SCIENCE, John Wiley & Sons, Inc. 1995-1997; 5 Stewart and Young, 1984, Solid Phase Peptide Synthesis, 2nd ed. Pierce Chemical Co., Rockford, Ill; and Atherton and Shephard, *supra*).

Alternatively, individual peptides may be prepared by a procedure including the steps of: (a) preparing a synthetic construct including a synthetic polynucleotide encoding an individual peptide of an overlapping set of peptides, wherein the synthetic polynucleotide is operably linked to a regulatory polynucleotide; (b) introducing the 10 synthetic construct into a suitable host cell; (c) culturing the host cell to express the synthetic polynucleotide; and (d) isolating the individual peptide. The synthetic construct is preferably in the form of an expression vector. For example, the expression vector can be a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into 15 a host genome. Typically, the regulatory polynucleotide includes, but is not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring 20 promoters, or hybrid promoters that combine elements of more than one promoter. The regulatory polynucleotide will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory polynucleotides are known in the art for a variety of host cells. In certain embodiments, the expression vector contains a selectable marker gene to allow the selection of transformed 25 host cells. Selection genes are well known in the art and will vary with the host cell used. In other embodiments, the expression vector also includes a nucleic acid sequence that codes for a fusion partner so that an individual peptide is expressed as a fusion polypeptide with the fusion partner. The main advantage of fusion partners is that they assist identification and/or purification of the fusion polypeptide. Exemplary fusion partners 30 include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS₆), which are particularly useful for isolation of the fusion polypeptide by affinity chromatography. For the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices for affinity

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chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS₆) fusion partners and the Pharmacia GST purification system. In a preferred embodiment, the recombinant polynucleotide is expressed in the commercial vector pFLAG™. Advantageously, the fusion partners also have protease cleavage sites, such as for Factor X_a, Thrombin and inteins (protein introns), which allow the relevant protease to partially digest the fusion polypeptide of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated peptide can then be isolated from the fusion partner by subsequent chromatographic separation. Fusion partners according to the invention also include within their scope "epitope tags", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-Myc, influenza virus, haemagglutinin and FLAG tags.

The step of introducing the synthetic construct into the host cell may be achieved using any suitable technique including transfection, and transformation, the choice of which will be dependent on the host cell employed. Such methods are well known to those of skill in the art. The peptides of the invention may be produced by culturing a host cell transformed with the synthetic construct. The conditions appropriate for protein expression will vary with the choice of expression vector and the host cell. This is easily ascertained by one skilled in the art through routine experimentation. Suitable host cells for expression may be prokaryotic or eukaryotic. One preferred host cell for expression of a polypeptide according to the invention is a bacterium. The bacterium used may be *Escherichia coli*. Alternatively, the host cell may be an insect cell such as, for example, *SF9* cells that may be utilised with a baculovirus expression system.

The amino acids of the peptides can be any non-naturally occurring or any naturally occurring amino acid. Examples of unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminoheptanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated by the present invention is shown in TABLE B.

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TABLE B

| <i>Non-conventional amino acid</i> | <i>Non-conventional amino acid</i> |
|---|------------------------------------|
| α -aminobutyric acid | L-N-methylalanine |
| α -amino- α -methylbutyrate | L-N-methylarginine |
| aminocyclopropane-carboxylate | L-N-methylasparagine |
| aminoisobutyric acid | L-N-methylaspartic acid |
| aminonorbornyl-carboxylate | L-N-methylcysteine |
| cyclohexylalanine | L-N-methylglutamine |
| cyclopentylalanine | L-N-methylglutamic acid |
| L-N-methylisoleucine | L-N-methylhistidine |
| D-alanine | L-N-methylleucine |
| D-arginine | L-N-methyllysine |
| D-aspartic acid | L-N-methylmethionine |
| D-cysteine | L-N-methylnorleucine |
| D-glutamate | L-N-methylnorvaline |
| D-glutamic acid | L-N-methylornithine |
| D-histidine | L-N-methylphenylalanine |
| D-isoleucine | L-N-methylproline |
| D-leucine | L-N-methylserine |
| D-lysine | L-N-methylthreonine |
| D-methionine | L-N-methyltryptophan |
| D-ornithine | L-N-methyltyrosine |
| D-phenylalanine | L-N-methylvaline |
| D-proline | L-N-methylethylglycine |
| D-serine | L-N-methyl-t-butylglycine |
| D-threonine | L-norleucine |

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| <i>Non-conventional amino acid</i> | <i>Non-conventional amino acid</i> |
|------------------------------------|---|
| D-tryptophan | L-norvaline |
| D-tyrosine | α -methyl-aminoisobutyrate |
| D-valine | α -methyl- γ -aminobutyrate |
| D- α -methylalanine | α -methylcyclohexylalanine |
| D- α -methylarginine | α -methylcyclopentylalanine |
| D- α -methylasparagine | α -methyl- α -naphthylalanine |
| D- α -methylaspartate | α -methylpenicillamine |
| D- α -methylcysteine | N-(4-aminobutyl)glycine |
| D- α -methylglutamine | N-(2-aminoethyl)glycine |
| D- α -methylhistidine | N-(3-aminopropyl)glycine |
| D- α -methylisoleucine | N-amino- α -methylbutyrate |
| D- α -methylleucine | α -naphthylalanine |
| D- α -methyllysine | N-benzylglycine |
| D- α -methylmethionine | N-(2-carbamylethyl)glycine |
| D- α -methylornithine | N-(carbamylmethyl)glycine |
| D- α -methylphenylalanine | N-(2-carboxyethyl)glycine |
| D- α -methylproline | N-(carboxymethyl)glycine |
| D- α -methylserine | N-cyclobutylglycine |
| D- α -methylthreonine | N-cycloheptylglycine |
| D- α -methyltryptophan | N-cyclohexylglycine |
| D- α -methyltyrosine | N-cyclodecylglycine |
| L- α -methylleucine | L- α -methyllysine |
| L- α -methylmethionine | L- α -methylnorleucine |
| L- α -methylnorvaline | L- α -methylornithine |
| L- α -methylphenylalanine | L- α -methylproline |

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| <i>Non-conventional amino acid</i> | <i>Non-conventional amino acid</i> |
|---|--|
| L- α -methylserine | L- α -methylthreonine |
| L- α -methyltryptophan | L- α -methyltyrosine |
| L- α -methylvaline | L-N-methylhomophenylalanine |
| N-(N-(2,2-diphenylethyl carbamylmethyl)glycine | N-(N-(3,3-diphenylpropyl carbamylmethyl)glycine |
| 1-carboxy-1-(2,2-diphenyl-ethyl amino)cyclopropane | |

The invention also contemplates modifying the peptides of the invention using ordinary molecular biological techniques so as to alter their resistance to proteolytic degradation or to optimise solubility properties or to render them more suitable as an immunogenic agent.

3. *Antigen-presenting cell embodiments*

The present invention is predicated in part on the determination that antigen-presenting cells which have been contacted with overlapping peptide sets as described in Section 2 are potent modulators of immune responses and are especially useful for raising strong immunogenic responses that can prevent or ameliorate the symptoms of a disease or condition of interest. Accordingly, the invention provides a process for producing antigen-specific antigen-presenting cells, comprising contacting antigen-presenting cells or their precursors with one or more sets of peptides as broadly described above for a time and under conditions sufficient for the peptides or processed forms thereof to be presented by the antigen-presenting cells or their precursors, and in the case of precursors, culturing the precursors for a time and under conditions sufficient to differentiate antigen-presenting cells from the precursors.

3.1 Sources of antigen-presenting cells

The antigen-presenting cells suitably encompasses both professional and facultative types of antigen-presenting cells. For example, professional antigen-presenting cells include, but are not limited to, macrophages, monocytes, cells of myeloid lineage, including monocytic-granulocytic-DC precursors, marginal zone Kupffer cells, microglia, T cells, B cells Langerhans cells and dendritic cells including interdigitating dendritic cells

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and follicular dendritic cells. Examples of facultative antigen-presenting cells include but are not limited to activated T cells, astrocytes, follicular cells, endothelium and fibroblasts. In a preferred embodiment, the antigen-presenting cells are selected from monocytes, macrophages, cells of myeloid lineage, dendritic cells or Langerhans cells.

5 Antigen-presenting cells or their precursors can be isolated by methods known to those of skill in the art. The source of antigen-presenting cell or precursor will differ depending upon the antigen-presenting cell required for modulating a specified immune response. In this context, the antigen-presenting cell can be selected from dendritic cells, macrophages, monocytes and other cells of myeloid lineage. Typically, precursors of
10 antigen-presenting cells can be isolated from any tissue, but are most easily isolated from blood, cord blood or bone marrow (Sorg *et al.*, 2001, *Exp Hematol* 29: 1289-1294; Zheng *et al.*, 2000, *J Hematother Stem Cell Res* 9: 453-464). It is also possible to obtain suitable precursors from diseased tissues such as rheumatoid synovial tissue or fluid following biopsy or joint tap (Thomas *et al.*, 1994, *J Immunol* 152: 2613-2623; Thomas *et al.*, 1994,
15 *J Immunol* 153: 4016-4028). Other examples include, but are not limited to liver, spleen, heart, kidney, gut and tonsil (Lu *et al.*, 1994, *Transplantation* 64: 1808-1815; McIlroy *et al.*, 2001, *Blood* 97: 3470-3477; Vremec *et al.*, 2000, *J Immunol* 164: 2978-2986; Hart and Fabre, 1981, *J Exp Med* 154(2): 347-361; Hart and McKenzie, 1988, *J Exp Med* 168(1): 157-170; Pavli *et al.*, 1990, *Immunology* 70(1): 40-47).

20 Leukocytes isolated directly from tissue provide a major source of antigen-presenting cell precursors. Typically, these precursors can only differentiate into antigen-presenting cells by culturing in the presence or absence of various growth factors. According to the practice of the present invention, the antigen-presenting cells may be so differentiated from crude mixtures or from partially or substantially purified preparations
25 of precursors. Leukocytes can be conveniently purified from blood or bone marrow by density gradient centrifugation using, for example, Ficoll Hypaque which eliminates neutrophils and red cells (peripheral blood mononuclear cells or PBMCs), or by ammonium chloride lysis of red cells (leukocytes or white blood cells). Many precursors of antigen-presenting cells are present in peripheral blood as non-proliferating monocytes,
30 which can be differentiated into specific antigen-presenting cells, including macrophages and dendritic cells, by culturing in the presence of specific cytokines.

Tissue-derived precursors such as precursors of tissue dendritic cells or of Langerhans cells are typically obtained by mincing tissue (e.g., basal layer of epidermis)

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and digesting it with collagenase or dispase followed by density gradient separation, or selection of precursors based on their expression of cell surface markers. For example, Langerhans cell precursors express CD1 molecules as well as HLA-DR and can be purified on this basis.

5 In one embodiment, the antigen-presenting cell precursor is a precursor of macrophages. Generally these precursors can be obtained from monocytes of any source and can be differentiated into macrophages by prolonged incubation in the presence of medium and macrophage colony stimulating factor (M-CSF) (Erickson-Miller *et al.*, 1990, *Int J Cell Cloning* 8: 346-356; Metcalf and Burgess, 1982, *J Cell Physiol* 111: 275-283).

10 In another embodiment, the antigen presenting cell precursor is a precursor of Langerhans cells. Usually, Langerhans cells can be generated from human monocytes or CD34⁺ bone marrow precursors in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-4/TNF α and TGF β (Geissmann *et al.*, 1998, *J Exp Med* 187: 961-966; Strobl *et al.*, 1997, *Blood* 90: 1425-1434 Strobl *et al.*, 1997, *Adv Exp Med*
15 *Biol* 417: 161-165; Strobl *et al.*, 1996, *J Immunol* 157: 1499-1507).

 In one embodiment, the antigen-presenting cell precursor is a precursor of dendritic cells. Several potential dendritic cell precursors can be obtained from peripheral blood, cord blood or bone marrow. These include monocytes, CD34⁺ stem cells, granulocytes, CD33⁺CD11c⁺ DC precursors, and committed myeloid progenitors –
20 described below.

Monocytes. Monocytes can be purified by adherence to plastic for 1-2 h in the presence of tissue culture medium (e.g., RPMI) and serum (e.g., human or foetal calf serum), or in serum-free medium (Anton *et al.*, 1998, *Scand J Immunol* 47: 116-121.; Araki *et al.*, 2001, *Br J Haematol* 114: 681-689; Mackensen *et al.*, 2000, *Int J Cancer* 86:
25 385-392; Nestle *et al.*, 1998, *Nat Med* 4: 328-332; Romani *et al.*, 1996, *J Immunol Meth* 196: 137-151; Thurner *et al.*, 1999, *J Immunol Methods* 223: 1-15). Monocytes can also be elutriated from peripheral blood (Garderet *et al.*, 2001, *J Hematother Stem Cell Res* 10: 553-567). Monocytes can also be purified by immunoaffinity techniques, including immunomagnetic selection, flow cytometric sorting or panning (Araki *et al.*, 2001, *supra*;
30 Battye and Shortman, 1991, *Curr. Opin. Immunol.* 3: 238-241), with anti-CD14 antibodies to obtain CD14^{hi} cells. The numbers (and therefore yield) of circulating monocytes can be enhanced by the *in vivo* use of various cytokines including GM-CSF (Groopman *et al.*,

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1987; *N Engl J Med* 317: 593-598; Hill *et al.*, 1995, *J Leukoc Biol* 58: 634-642). Monocytes can be differentiated into dendritic cells by prolonged incubation in the presence of GM-CSF and IL-4 (Romani *et al.*, 1994, *J Exp Med* 180: 83-93; Romani *et al.*, 1996, *supra*). A combination of GM-CSF and IL-4 at a concentration of each at between
5 about 200 to about 2000 U/mL, more preferably between about 500 to about 1000 U/mL and even more preferably between about 800 U/mL (GM-CSF) and 1000 U/mL (IL-4) produces significant quantities of immature dendritic cells, *i.e.*, antigen-capturing phagocytic dendritic cells. Other cytokines which promote differentiation of monocytes into antigen-capturing phagocytic dendritic cells include, for example, IL-13.

10 **CD34⁺ stem cells.** Dendritic cells can also be generated from CD34⁺ bone marrow derived precursors in the presence of GM-CSF, TNF α \pm stem cell factor (SCF, c-kitL), or GM-CSF, IL-4 \pm flt3L (Bai *et al.*, 2002, *Int J Oncol* 20: 247-53; Chen *et al.*, 2001, *Clin Immunol* 98: 280-292; Loudovaris *et al.*, 2001, *J Hematother Stem Cell Res* 10: 569-578). CD34⁺ cells can be derived from a bone marrow aspirate or from blood and can be
15 enriched as for monocytes using, for example, immunomagnetic selection or immunocolumns (Davis *et al.*, 1994, *J Immunol Meth* 175: 247-257). The proportion of CD34⁺ cells in blood can be enhanced by the *in vivo* use of various cytokines including (most commonly) G-CSF, but also flt3L and progenipoiectin (Fleming *et al.*, 2001, *Exp Hematol* 29: 943-951; Pulendran *et al.*, 2000, *J Immunol* 165: 566-572; Robinson *et al.*,
20 2000, *J Hematother Stem Cell Res* 9: 711-720).

Other myeloid progenitors. DC can be generated from committed early myeloid progenitors in a similar fashion to CD34⁺ stem cells, in the presence of GM-CSF and IL-4/TNF. Such myeloid precursors infiltrate many tissues in inflammation, including rheumatoid arthritis synovial fluid (Santiago-Schwarz *et al.*, 2001, *J Immunol* 167(3):
25 1758-68). Expansion of total body myeloid cells including circulating dendritic cell precursors and monocytes, can be achieved with certain cytokines, including flt-3 ligand, granulocyte colony-stimulating factor (G-CSF) or progenipoiectin (pro-GP) (Fleming *et al.*, 2001, *supra*; Pulendran *et al.*, 2000, *supra*; Robinson *et al.*, 2000, *supra*). Administration of such cytokines for several days to a human or other mammal would enable much larger
30 numbers of precursors to be derived from peripheral blood or bone marrow for *in vitro* manipulation. Dendritic cells can also be generated from peripheral blood neutrophil precursors in the presence of GM-CSF, IL-4 and TNF α (Kelly *et al.*, 2001, *Cell Mol Biol (Noisy-le-grand)* 47(1): 43-54; Oehler *et al.*, 1998, *J Exp Med.* 187(7):1019-28). It should

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be noted that dendritic cells can also be generated, using similar methods, from acute myeloid leukemia cells (Oehler *et al.*, 2000, *Ann Hematol* 79(7): 355-62).

Tissue DC precursors and other sources of APC precursors. Other methods for DC generation exist from, for example, thymic precursors in the presence of IL-3 +/- GM-CSF, and liver DC precursors in the presence of GM-CSF and a collagen matrix. Transformed or immortalised dendritic cell lines may be produced using oncogenes such as *v-myc* as for example described by (Paglia *et al.*, 1993, *J Exp Med* 178(6): 1893-901) or by *myb* (Banyer and Hapel, 1999, *J Leukoc Biol* 66(2): 217-223; Gonda *et al.*, 1993, *Blood* 82(9): 2813-2822).

Circulating DC precursors. These have been described in human and mouse peripheral blood. One can also take advantage of particular cell surface markers for identifying suitable dendritic cell precursors. Specifically, various populations of dendritic cell precursors can be identified in blood by the expression of CD11c and the absence or low expression of CD14, CD19, CD56 and CD3 (O'Doherty *et al.*, 1994, *Immunology* 82: 487-493; O'Doherty *et al.*, 1993, *J Exp Med* 178: 1067-1078). These cells can also be identified by the cell surface markers CD13 and CD33 (Thomas *et al.*, 1993, *J Immunol* 151(12): 6840-6852). A second subset, which lacks CD14, CD19, CD56 and CD3, known as plasmacytoid dendritic cell precursors, does not express CD11c, but does express CD123 (IL-3R chain) and HLA-DR (Farkas *et al.*, 2001, *Am J Pathol* 159: 237-243; Grouard *et al.*, 1997, *J Exp Med* 185: 1101-1111; Rissoan *et al.*, 1999, *Science* 283: 1183-1186). Most circulating CD11c⁺ dendritic cell precursors are HLA-DR⁺, however some precursors may be HLA-DR⁻. The lack of MHC class II expression has been clearly demonstrated for peripheral blood dendritic cell precursors (del Hoyo *et al.*, 2002, *Nature* 415: 1043-1047).

Optionally, CD33⁺CD14^{-/lo} or CD11c⁺HLA-DR⁺, lineage marker-negative dendritic cell precursors described above can be differentiated into more mature antigen-presenting cells by incubation for 18-36 h in culture medium or in monocyte conditioned medium (Thomas *et al.*, 1993, *J Immunol* 151(12): 6840-6852; Thomas and Lipsky, 1994, *J Immunol* 153: 4016-4028; O'Doherty *et al.*, 1993, *supra*). Alternatively, following incubation of peripheral blood non-T cells or unpurified PBMC, the mature peripheral blood dendritic cells are characterised by low density and so can be purified on density gradients, including metrizamide and Nycodenz (Freudenthal and Steinman, 1990, *Proc Natl Acad Sci U S A* 87: 7698-7702; Vremec and Shortman, 1997, *J Immunol* 159: 565-

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573), or by specific monoclonal antibodies, such as but not limited to the CMRF-44 mAb (Fearnley *et al.*, 1999, *Blood* 93, 728-736; Vuckovic *et al.*, 1998, *Exp Hematol* 26: 1255-1264). Plasmacytoid dendritic cells can be purified directly from peripheral blood on the basis of cell surface markers, and then incubated in the presence of IL-3 (Grouard *et al.*, 1997, *supra*; Rissoan *et al.*, 1999, *supra*). Alternatively, plasmacytoid DC can be derived from density gradients or CMRF-44 selection of incubated peripheral blood cells as above.

In general, for dendritic cells generated from any precursor, when incubated in the presence of activation factors such as monocyte-derived cytokines, lipopolysaccharide and DNA containing CpG repeats, cytokines such as TNF- α , IL-6, IFN- α , IL-1 β , necrotic cells, readherence, whole bacteria, membrane components, RNA or polyIC, immature dendritic cells will become activated (Clark, 2002, *J Leukoc Biol* 71: 388-400; Hacker *et al.*, 2002, *Immunology* 105: 245-251; Kaisho and Akira, 2002, *Biochim Biophys Acta* 1589: 1-13; Koski *et al.*, 2001, *Crit Rev Immunol* 21: 179-189).

Other methods for isolation, expansion and/or maturation of dendritic cells for the purpose of the present invention are described for example by Takamizawa *et al.* (1997, *J Immunol*, 158(5): 2134-2142), Thomas and Lipsky (1994, *J Immunol*, 153(9): 4016-4028), O'Doherty *et al.* (1994, *Immunology*, 82(3): 487-93), Fearnley *et al.* (1997, *Blood*, 89(10): 3708-3716), Weissman *et al.* (1995, *Proc Natl Acad Sci U S A*, 92(3): 826-830), Freudenthal and Steinman (1990, *Proc Natl Acad Sci U S A*, 87(19): 7698-7702), Romani *et al.* (1996, *J Immunol Methods*, 196(2): 137-151), Reddy *et al.* (1997, *Blood*, 90(9): 3640-3646), Thurnher *et al.* (1997, *Exp Hematol*, 25(3): 232-237), Caux *et al.* (1996, *J Exp Med*, 184(2): 695-706; 1996, *Blood*, 87(6): 2376-85), Luft *et al.* (1998, *Exp Hematol*, 26(6): 489-500; 1998, *J Immunol*, 161(4): 1947-1953), Cella *et al.* (1999, *J Exp Med*, 189(5): 821-829; 1997, *Nature*, 388(644): 782-787; 1996, *J Exp Med*, 184(2): 747-572), Ahonen *et al.* (1999, *Cell Immunol*, 197(1): 62-72) and Piemonti *et al.* (1999, *J Immunol*, 162(11): 6473-6481).

In some embodiments, the antigen-presenting cells are in the form of a substantially purified population of cells. In other embodiments, the antigen-presenting cells are in the form of a heterogeneous pool of cells, which is suitably selected from blood and peripheral blood mononuclear cells (PBMC). This latter embodiment is particularly advantageous because it avoids the need to obtain homogeneous populations of cells, which is generally a time-consuming and costly process. The present inventors have also discovered that it is possible to contact such heterogeneous populations with the

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overlapping peptides of the invention for short time periods (e.g., as low as 5, 10, 15, 20, 20, 40, 50, 60 min) and infusing the contacted cells directly into a recipient without further culturing of the cells. This further shortens the processing time to permit potentially the harvesting of autologous or syngeneic antigen-presenting cells, treatment of those cells with peptide and infusion of the peptide-contacted cells into a patient in a single sitting or day.

3.2 Delivery of peptide antigen into antigen-presenting cells

The delivery of exogenous peptide antigen to antigen-presenting cells can be enhanced by methods known to practitioners in the art. For example, several different strategies have been developed for delivery of exogenous antigen to the endogenous processing pathway of antigen-presenting cells, especially dendritic cells. These methods include insertion of antigen into pH-sensitive liposomes (Zhou and Huang, 1994, *Immunomethods*, 4:229-235), osmotic lysis of pinosomes after pinocytic uptake of soluble antigen (Moore *et al.*, 1988, *Cell*, 54:777-785), coupling of antigens to potent adjuvants (Aichele *et al.*, 1990, *J. Exp. Med.*, 171: 1815-1820; Gao *et al.*, 1991, *J. Immunol.*, 147: 3268-3273; Schulz *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88: 991-993; Kuzu *et al.*, 1993, *Euro. J. Immunol.*, 23: 1397-1400; and Jondal *et al.*, 1996, *Immunity* 5: 295-302) and apoptotic cell delivery of antigen (Albert *et al.* 1998, *Nature* 392:86-89; Albert *et al.* 1998, *Nature Med.* 4:1321-1324; and in International Publications WO 99/42564 and WO 01/85207). Recombinant bacteria (eg. *E. coli*) or transfected host mammalian cells may be pulsed onto dendritic cells (as particulate antigen, or apoptotic bodies respectively) for antigen delivery. Recombinant chimeric virus-like particles (VLPs) have also been used as vehicles for delivery of exogenous heterologous antigen to the MHC class I processing pathway of a dendritic cell line (Bachmann *et al.*, 1996, *Eur. J. Immunol.*, 26(11): 2595-2600).

Alternatively, or in addition, a peptide antigen may be linked to, or otherwise associated with, a cytolysin to enhance the transfer of the peptide into the cytosol of an antigen-presenting cell of the invention for delivery to the MHC class I pathway. Exemplary cytolysins include saponin compounds such as saponin-containing Immune Stimulating Complexes (ISCOMs) (see e.g., Cox and Coulter, 1997, *Vaccine* 15(3): 248-256 and U.S. Patent No. 6,352,697), phospholipases (see, e.g., Camilli *et al.*, 1991, *J. Exp. Med.* 173: 751-754), pore-forming toxins (e.g., an alpha-toxin), natural cytolysins of gram-

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positive bacteria, such as listeriolysin O (LLO, e.g., Mengaud *et al.*, 1988, *Infect. Immun.* 56: 766-772 and Portnoy *et al.*, 1992, *Infect. Immun.* 60: 2710-2717), streptolysin O (SLO, e.g., Palmer *et al.*, 1998, *Biochemistry* 37(8): 2378-2383) and perfringolysin O (PFO, e.g., Rossjohn *et al.*, *Cell* 89(5): 685-692). Where the antigen-presenting cell is phagosomal, acid activated cytolysins may be advantageously used. For example, listeriolysin exhibits greater pore-forming ability at mildly acidic pH (the pH conditions within the phagosome), thereby facilitating delivery of vacuole (including phagosome and endosome) contents to the cytoplasm (see, e.g., Portnoy *et al.*, *Infect. Immun.* 1992, 60: 2710-2717).

The amount of peptide antigen to be placed in contact with antigen-presenting cells can be determined empirically by persons of skill in the art. The antigen-presenting cells should be exposed to the peptides for a period of time sufficient for those cells to present the peptides or processed forms thereof for the modulation of T cells. The time and dose of peptides necessary for the cells to optionally process and present the peptides or their processed forms may be determined using pulse-chase protocols in which exposure to peptides is followed by a washout period and exposure to a read-out system e.g., antigen reactive T cells. Once the optimal time and dose necessary for cells to express the peptides or their processed forms on their surface is determined, a protocol may be used to prepare cells and peptides for inducing immunogenic responses. Those of skill in the art will recognise in this regard that the length of time necessary for an antigen-presenting cell to present an antigen on its surface may vary depending on the antigen or form of antigen employed, its dose, and the antigen-presenting cell employed, as well as the conditions under which antigen loading is undertaken. These parameters can be determined by the skilled artisan using routine procedures. Efficiency of priming of the antigen-presenting cells can be determined by assaying T cell cytotoxic activity *in vitro* or using antigen-presenting cells as targets of CTLs. Other methods known to practitioners in the art, which can detect the presence of antigen on the surface of antigen-presenting cells after exposure to one or more of the modified and unmodified antigens, are also contemplated by the presented invention.

Usually, for peptides, about 0.1 to 20 µg/mL to about 1-10 million antigen-presenting cells, is suitable for producing primed antigen-specific antigen-presenting cells. Typically antigen-presenting cells are incubated with peptide for about 1 to 6 hr at a suitably temperature that permits processing and/or presentation of antigen by the antigen-presenting cells (e.g., at about 4, 6, 8, 10, 12, 16° C and desirably at 20, 25, 30, 37° C),

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although it is also possible to expose antigen-presenting cells to antigen for the duration of incubation with growth factors and inhibitor. Surprisingly, the present inventors have shown that successful presentation of peptides or their processed forms can be achieved using much shorter periods of incubation (e.g., about 5, 10, 15, 20, 30, 40, 50 minutes) using peptides at a concentration of about 10-20 µg/mL.

4. *Lymphocyte embodiments*

The antigen-presenting cells of the invention may be obtained or prepared to contain and/or express one or more antigens by any number of means, such that the antigen(s) or processed form(s) thereof, is (are) presented by those cells for potential modulation of other immune cells, including T lymphocytes and B lymphocytes, and particularly for producing T lymphocytes and B lymphocytes that are primed to respond to a specified antigen or group of antigens. In some embodiments, the subject antigen-presenting cells are useful for producing primed T lymphocytes to an antigen or group of antigens. The efficiency of inducing lymphocytes, especially T lymphocytes, to exhibit an immune response to a specified antigen can be determined by any suitable method including, but not limited to, assaying T lymphocyte cytolytic activity *in vitro* using for example antigen-specific antigen-presenting cells as targets of antigen-specific cytolytic T lymphocytes (CTL); assaying antigen-specific T lymphocyte proliferation (see, e.g., Vollenweider and Groseurth, 1992, *J. Immunol. Meth.* 149: 133-135), measuring B cell response to the antigen using, for example, ELISPOT assays, and ELISA assays; interrogating cytokine profiles; or measuring delayed-type hypersensitivity (DTH) responses by test of skin reactivity to a specified antigen (see, e.g., Chang *et al.* (1993, *Cancer Res.* 53: 1043-1050). Other methods known to practitioners in the art, which can detect the presence of antigen on the surface of antigen-presenting cells after exposure to the antigen, are also contemplated by the present invention.

Accordingly, the present invention also provides antigen-specific B or T lymphocytes, especially T lymphocytes, which respond in an antigen-specific fashion to representation of the antigen. In some embodiments, antigen-specific T lymphocytes are produced by contacting an antigen-presenting cell as defined above with a population of T lymphocytes, which may be obtained from any suitable source such as spleen or tonsil/lymph nodes but is preferably obtained from peripheral blood. The T lymphocytes can be used as crude preparations or as partially purified or substantially purified

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preparations, which are suitably obtained using standard techniques as, for example, described in "Immunochemical Techniques, Part G: Separation and Characterization of Lymphoid Cells" (*Meth. in Enzymol.* 108, Edited by Di Sabato *et al.*, 1984, Academic Press). This includes rosetting with sheep red blood cells, passage across columns of nylon wool or plastic adherence to deplete adherent cells, immunomagnetic or flow cytometric selection using appropriate monoclonal antibodies is known in the art.

The preparation of T lymphocytes is contacted with the antigen-presenting cells of the invention for an adequate period of time for priming the T lymphocytes to the antigen or antigens presented by those antigen-presenting cells. This period will preferably be at least about 1 day, and up to about 5 days.

In some embodiments, a population of antigen-presenting cells is cultured in the presence of a heterogeneous population of T lymphocytes, which is suitably obtained from peripheral blood, together with a set of peptides of the invention corresponding to an antigen to which an immune response is required. These cells are cultured for a period of time and under conditions sufficient for the peptides, or their processed forms, to be presented by the antigen-presenting cells; and the antigen-presenting cells to prime a subpopulation of the T lymphocytes to respond to the antigen.

5. Cell based therapy or prophylaxis

The antigen-presenting cells described in Section 3 and the lymphocytes described in Section 4 can be administered to a patient, either by themselves or in combination, for modulating an immune response, especially for priming an immune response to one or more cognate antigens. These cell based compositions are useful, therefore, for treating or preventing a disease or condition as noted above. The cells of the invention can be introduced into a patient by any means (*e.g.*, injection), which produces the desired immune response to an antigen or group of antigens. The cells may be derived from the patient (*i.e.*, autologous cells) or from an individual or individuals who are MHC matched or mismatched (*i.e.*, allogeneic) with the patient. Typically, autologous cells are injected back into the patient from whom the source cells were obtained. The injection site may be subcutaneous, intraperitoneal, intramuscular, intradermal, or intravenous. The cells may be administered to a patient already suffering from a disease or condition or who is predisposed to a disease or condition in sufficient number to treat or prevent or alleviate the symptoms of the disease or condition. The number of cells injected into the patient in

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need of the treatment or prophylaxis may vary depending on *inter alia*, the antigen or antigens and size of the individual. This number may range for example between about 10^3 and 10^{11} , and usually between about 10^5 and 10^7 cells (e.g., dendritic cells or T lymphocytes). Single or multiple administrations of the cells can be carried out with cell numbers and pattern being selected by the treating physician. The cells should be administered in a pharmaceutically acceptable carrier, which is non-toxic to the cells and the individual. Such carrier may be the growth medium in which the cells were grown, or any suitable buffering medium such as phosphate buffered saline. The cells may be administered alone or as an adjunct therapy in conjunction with other therapeutics known in the art for the treatment or prevention of unwanted immune responses for example but not limited to glucocorticoids, methotrexate, D-penicillamine, hydroxychloroquine, gold salts, sulfasalazine, TNF α or interleukin-1 inhibitors, and/or other forms of specific immunotherapy.

6. Compositions

The overlapping sets of peptides described in Sections 2 and the antigen-primed antigen-presenting cells described in Section 3 or the lymphocytes described in Section 4 (therapeutic/prophylactic agents) can be used singly or together as active ingredients for the treatment or prophylaxis of various conditions associated with the presence of one or more target polypeptide antigens. These therapeutic/prophylactic agents can be administered to a patient either by themselves, or in compositions where they are mixed with a suitable pharmaceutically acceptable carrier and/or diluent, or an adjuvant.

The invention also encompasses a method for stimulating a patient's immune system, and preferably for eliciting a humoral and/or cellular immune response to a polypeptide of interest, by administering to the patient a therapeutic agent or composition as described above. Such stimulation may be utilised for the treatment and/or prophylaxis of a disease or condition including, but not restricted to, a pathogenic infection (e.g., viral, bacterial, fungal, protozoan) or a cancer. Accordingly, the invention contemplates a method for treatment and/or prophylaxis of a disease or condition, comprising administering to a patient in need of such treatment a therapeutically/prophylactically effective amount of a therapeutic agent or composition as broadly described above.

Depending on the specific conditions being treated, therapeutic/prophylactic agents may be formulated and administered systemically or locally. Techniques for

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formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition. Suitable routes may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct
5 intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. For injection, which constitutes one desirable embodiment of the present invention, the therapeutic agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the
10 barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines. In certain embodiments of the present invention, the immunogenic compositions are administered intravenously.

15 The therapeutic/prophylactic agents can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated in dosage forms such as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. These carriers may be selected from
20 sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulphate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to
25 achieve its intended purpose. The dose of agent administered to a patient should be sufficient to effect a beneficial response in the patient over time such as a reduction in the symptoms associated with the condition. The quantity of the therapeutic/prophylactic agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof. In this regard, precise amounts of the
30 therapeutic/prophylactic agent(s) for administration will depend on the judgement of the practitioner. In determining the effective amount of the agent to be administered in the treatment or prophylaxis of the condition, the physician may evaluate tissue levels of a

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target antigen, and progression of the disease or condition. In any event, those of skill in the art may readily determine suitable dosages of the therapeutic agents of the invention.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilisers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as., for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more therapeutic agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilising processes.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterise different combinations of active compound doses.

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Pharmaceutical which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticiser, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilisers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilisers may be added.

Dosage forms of the therapeutic agents of the invention may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of an agent of the invention may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Therapeutic agents of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulphuric, acetic, lactic, tartaric, malic, succinic, *etc.* Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

For any compound used in the method of the invention, the effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC₅₀ as determined in cell culture (e.g., the concentration of a test agent, which achieves a half-maximal reduction in target antigen). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the compounds of the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit large therapeutic indices are preferred. The data

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obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilised. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See for example Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active compound(s) which are sufficient to maintain target antigen-reducing effects or effects that ameliorate the disease or condition. Usual patient dosages for systemic administration range from 1-2000 mg/day, commonly from 1-250 mg/day, and typically from 10-150 mg/day. Stated in terms of patient body weight, usual dosages range from 0.02-25 mg/kg/day, commonly from 0.02-3 mg/kg/day, typically from 0.2-1.5 mg/kg/day. Stated in terms of patient body surface areas, usual dosages range from 0.5-1200 mg/m²/day, commonly from 0.5-150 mg/m²/day, typically from 5-100 mg/m²/day.

Alternately, one may administer the agent in a local rather than systemic manner, for example, *via* injection of the compound directly into a tissue, often in a depot or sustained release formulation. Furthermore, one may administer the agent in a targeted drug delivery system, for example, in a liposome coated with tissue-specific antibody. The liposomes will be targeted to and taken up selectively by the tissue.

From the foregoing, it will be appreciated that the agents of the invention may be used as therapeutic or prophylactic immunomodulating compositions or vaccines. Accordingly, the invention extends to the production of immunomodulating compositions containing as active compounds one or more of the therapeutic/prophylactic agents of the invention. Any suitable procedure is contemplated for producing such vaccines. Exemplary procedures include, for example, those described in NEW GENERATION VACCINES (1997, Levine *et al.*, Marcel Dekker, Inc. New York, Basel Hong Kong).

Immunomodulating compositions according to the present invention can contain a physiologically acceptable diluent or excipient such as water, phosphate buffered saline and saline. They may also include an adjuvant as is well known in the art. Suitable adjuvants include, but are not limited to: surface active substances such as hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin,

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dimethyldioctadecylammonium bromide, N, N-dioctadecyl-N', N'bis(2-hydroxyethyl-propanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines such as pyran, dextran sulfate, poly IC carbopol; peptides such as muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral gels such as aluminum phosphate, aluminum hydroxide or alum; lymphokines, QuilA and immune stimulating complexes (ISCOMS).

The antigen-primed antigen-presenting cells of the invention and antigen-specific T lymphocytes generated with these antigen-presenting cells, as described *infra*, can be used as active compounds in immunomodulating compositions for prophylactic or therapeutic applications. The primed cells, which are preferably mature dendritic cells, can be injected by any method that elicits an immune response into a syngeneic animal or human. Preferably, antigen-presenting cells are injected back into the same animal or human from whom the source tissue/cells was obtained. The injection site may be subcutaneous, intraperitoneal, intramuscular, intradermal, or intravenous. The number of antigen-primed antigen-presenting cells reinjected back into the animal or human in need of treatment may vary depending on *inter alia*, the antigen and size of the individual. This number may range for example between about 10^4 and 10^8 , and more preferably between about 10^6 and 10^7 antigen-primed antigen-presenting cells (e.g., dendritic cells). The antigen-presenting cells should be administered in a pharmaceutically acceptable carrier, which is non-toxic to the cells and the individual. Such carrier may be the growth medium in which the antigen-presenting cells were grown, or any suitable buffering medium such as phosphate buffered saline.

In one embodiment, the antigen-primed antigen-presenting cells of the invention could also be used for generating large numbers of CD8⁺ or CD4⁺ CTL, for adoptive transfer to immunosuppressed individuals who are unable to mount normal immune responses. For example, antigen-specific CD8⁺ CTL can be adoptively transferred for therapeutic purposes in individuals afflicted with HIV infection (Koup *et al.*, 1991, *J. Exp. Med.*, 174: 1593-1600; Carmichael *et al.*, 1993, *J. Exp. Med.*, 177: 249-256; and Johnson *et al.*, 1992, *J. Exp. Med.*, 175: 961-971), malaria (Hill *et al.*, 1992, *Nature*, 360: 434-439) and malignant tumours such as melanoma (Van der Brogen *et al.*, 1991, *Science*, 254: 1643-1647; and Young and Steinman, 1990, *J. Exp. Med.*, 171: 1315-1332).

In another embodiment, the immunomodulating composition of the invention is suitable for treatment or prophylaxis of a cancer. Cancers which could be suitably treated

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in accordance with the practices of this invention include cancers associated with a viral infection such as cervical cancer (e.g., papillomavirus infection) and Burkitt's lymphoma (e.g., Epstein Barr virus infection). Other virus associated cancers include, but are not restricted to, HTLV1 associated leukemia, Non Hodgkins lymphoma (EBV), anal cancer, skin cancer (HPV), hepatocellular carcinoma (HBV) and Kaposi sarcoma (HHV8). Alternatively, the cancer may be a non-virally associated cancer such as but not limited to melanoma, lung cancer, breast cancer, prostate cancer, colon cancer, pancreatic cancer, stomach cancer, bladder cancer, kidney cancer, post transplant lymphoproliferative disease (PTLD), Hodgkin's Lymphoma and the like.

In yet another embodiment, the immunomodulating composition is suitable for treatment or prophylaxis of a viral, bacterial or protozoan infection. Viral infections contemplated by the present invention include, but are not restricted to, infections caused by HIV, Hepatitis, Influenza, Japanese encephalitis virus, Epstein-Barr virus and respiratory syncytial virus. Bacterial infections include, but are not restricted to, those caused by *Neisseria* species, *Meningococcal* species, *Haemophilus* species *Salmonella* species, *Streptococcal* species, *Legionella* species and *Mycobacterium* species. Protozoan infections encompassed by the invention include, but are not restricted to, those caused by *Plasmodium* species (e.g., malaria), *Schistosoma* species (e.g., schistosomiasis), *Leishmania* species, *Trypanosoma* species, *Toxoplasma* species and *Giardia* species.

7. Methods for assessing immunomodulation

The effectiveness of the immunisation may be assessed using any suitable technique. An individual's capacity to respond to foreign or disease-specific antigens (e.g., viral antigens and cancer antigens) may be determined by assessing whether those cells primed to attack such antigens are increased in number, activity, and ability to detect and destroy those antigens. Strength of immune response is measured by standard tests including: direct measurement of peripheral blood lymphocytes by means known to the art; natural killer cell cytotoxicity assays (see, e.g., Provinciali M. *et al* (1992, *J. Immunol. Meth.* 155: 19-24), cell proliferation assays (see, e.g., Vollenweider, I. and Groseurth, P. J. (1992, *J. Immunol. Meth.* 149: 133-135), immunoassays of immune cells and subsets (see, e.g., Loeffler, D. A., *et al.* (1992, *Cytom.* 13: 169-174); Rivoltini, L., *et al.* (1992, *Can. Immunol. Immunother.* 34: 241-251); or skin tests for cell-mediated immunity (see, e.g., Chang, A. E. *et al* (1993, *Cancer Res.* 53: 1043-1050). Alternatively, the efficacy of the

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immunisation may be monitored using one or more techniques including, but not limited to, HLA class I tetramer staining - of both fresh and stimulated PBMCs (see for example Allen *et al.*, *supra*), proliferation assays (Allen *et al.*, *supra*), ELISPOT assays and intracellular cytokine staining (Allen *et al.*, *supra*), ELISA Assays - for linear B cell responses; and Western blots of cell sample expressing the synthetic polynucleotides. Particularly relevant will be the cytokine profile of T cells activated by antigen, and more particularly the production and secretion of IFN γ , IL-2, IL4, IL5, IL-10, TGF β and TNF α .

The cytotoxic activity of T lymphocytes, and in particular the ability of cytotoxic T lymphocytes to be induced by antigen-presenting cells, may be assessed by any suitable technique known to those of skill in the art. For example, a sample comprising T lymphocytes to be assayed for cytotoxic activity is obtained and the T lymphocytes are then exposed to antigen-primed antigen-presenting cells, which have been caused to present antigen. After an appropriate period of time, which may be determined by assessing the cytotoxic activity of a control population of T lymphocytes which are known to be capable of being induced to become cytotoxic cells, the T lymphocytes to be assessed are tested for cytotoxic activity in a standard cytotoxic assay.

The method of assessing CTL activity is particularly useful for evaluating an individual's capacity to generate a cytotoxic response against cells expressing tumour or viral antigens. Accordingly, this method is useful for evaluating an individual's ability to mount an immune response to a cancer or virus. For example, CTL lysis assays may be employed using stimulated splenocytes or peripheral blood mononuclear cells (PBMC) on peptide coated or recombinant virus infected cells using ^{51}Cr labelled target cells. Such assays can be performed using for example primate, mouse or human cells (Allen *et al.*, 2000, *J. Immunol.* 164(9): 4968-4978 also Woodberry *et al.*, *infra*).

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting experimental example.

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EXAMPLE

MATERIALS AND METHODS

Animals

18 Male juvenile, colony-bred pigtailed macaques (*Macaca nemestrina*, aged 2-4 years) were studied. All animals were housed under PC3 biosafety conditions by trained animal technicians at the CSIRO Australian Animal Health Laboratory, Geelong. Prior to all procedures, animals were anaesthetised with ketamine (10 mg/kg, intramuscularly). Health and weight were routinely monitored. All conditions and protocols were approved by the CSIRO animal health and the University of Melbourne animal ethics committees.

10 *Pre-immunisations*

To evaluate whether the OPAL method could boost T cell responses in animals with pre-primed responses. T cell responses were induced in macaques by administering 2 DNA vaccines expressing HIV or SIV structural genes followed by a FPV boost vaccine expressing similar HIV or SIV genes as previously described (16). DNA vaccines in saline were administered twice intramuscularly (0.5 ml to each anterior quadracep) at a dose of 1mg/dose. FPV boosts were delivered intramuscularly a dose of 5×10^7 pfu.

Isolation of plasma and peripheral blood mononuclear cells (PBMC) from whole blood

Blood was collected in 9ml Na+ Heparin and 3 mL EDTA vacutainers from the femoral vein of each animal on study weeks prior to and after vaccination and SHIV challenge. Plasma samples were removed following centrifugation (800×g, room temperature, RT, 8 min; Beckman Coulter) and stored in 3×1.5-mL tubes at -70° C. Plasma collected in EDTA-anticoagulated blood was used for RNA extraction. Media (RPMI-1640 supplemented with penicillin, streptomycin and glutamine; Invitrogen) equal to the volume of plasma collected was added to the blood and mixed prior to PBMC isolation on Ficoll-Paque, used according to the manufacturer's instructions (Amersham Pharmacia). PBMC were washed twice (500×g, 10° C, 6min) and resuspended in 1 mL media for counting (Beckman Coulter Counter®) in preparation of immunological assays.

Overlapping peptides

15-mer peptides (>80% purity) overlapping by 11 amino acids spanning the entire gag (125 peptides), pol (260 peptides) and nef (21 peptides) of SIV_{mac239} and env (211

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peptides) protein of SHIV_{SF162P3} (NIH AIDS Research and Reference Reagent depository) (Figure 1-4) were pooled for each protein by solubilising each 1mg peptide aliquot in 10-40 μ L of DMSO to final concentrations: SIV_{mac239} gag (670 μ g/ml or 730 μ g/ml); pol (304 μ g/ml), and; nef (4.762 mg/mL), and; SHIV_{SF162P3} env (330 μ g/mL), stored at -70°C until use.

SIV antigens for in vitro analyses

Whole inactivated SIV (WI SIV) and its control (supernatant from Hut78-CLE cell-line used to culture the WI SIV) (AIDS Vaccine Program, National Cancer Institute, MD) were stored at -70°C until use.

In vivo cytotoxic T lymphocyte killing

At weeks 10, 15 and 20 following the initial vaccination, PBMC from the macaques were isolated from 40-50 mL blood, as described above. 25 mL sterile injectable saline was infused into the animals immediately after blood sampling to prevent hypovolemia. PBMC were resuspended in PBS and divided into 3 or 4 equal volumes, 0.5ml. Cells were pulsed with SIVgag, pol, nef or SHIVenv peptide pools (10 μ g/mL) or DMSO (volume of equal to the volume of SIVgag), in PBS for 90 min at 37° C, or on ice, with regular mixing. To subsequently track each peptide-pulsed cell population by flow cytometry, each peptide/DMSO-pulsed population was then labelled with a concentration of CFSE or SNARF (Molecular Probes). 5mM CFSE stock in DMSO at -20° C was thawed and diluted in PBS. Neat SNARF stock was dissolved in 83 μ L DMSO to make 1mM and diluted in PBS. Table 1 shows the final concentrations of each dye. Cells were mixed thoroughly and stained for 10 min in a 37° C waterbath, followed by one wash in RF5 then PBS (500 \times g, 10°C, 6min). All peptide/DMSO-pulsed cells for each animal were pooled in 1.5ml saline for re-infusion into the femoral vein. 3ml blood was sampled from the opposite femoral vein at 5 min, and at 4 and 16hr following infusion. Red blood cells were lysed with 10ml FACS Lysing Solution (BD Biosciences), incubated for 10min at room temp. Cells were pelleted and washed twice with PBS (800 \times g, RT, 7min), and fixed with 1-2ml 2% paraformaldehyde (Figure 5).

To determine whether cell populations were being selectively killed, 10⁶ events gated live lymphocytes were collected by flow cytometry (FACSort Calibre, BD). CFSE and SNARF fluorescence were detected by FL1 and FL2 channels, respectively. For

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analysis, killing was expressed as the percentage of target versus control peptide-pulsed cell clearance. In the event of acquiring unequal labeled populations by flow cytometry at 5 minutes post-OPAL infusion, the degree of killing was subsequently scaled with respect to the initial population ratios obtained at 5 minutes. PBMC were also analysed prior to, and one week following, OPAL-infusion by IFN γ ELISpot and ICS to detect whether immune responses were enhanced.

SHIV challenge of macaques

To assess the efficacy to the vaccines, each macaque was inoculated intrarectally with SHIV_{mn229} (5×10^4 TCID₅₀/mL on CD8-depleted *M.nemestrina* PBMC) in 0.5 mL doses over 2 days (total 10^5 TCID₅₀/mL) 18 weeks after the initial immunisation, as previously described (32).

Quantification of viral SHIV RNA by reverse-transcriptase real-time PCR

RNA extraction: To detect SHIV RNA in macaques following SHIV challenge, total RNA was initially extracted from stored plasma samples from anti-coagulated blood collected in EDTA with QIAamp® Viral RNA commercial kit (Qiagen) as previously described (32). Briefly, plasma samples were centrifuged (500 \times g, RT, 10min) to remove cells (preventing DNA contamination). 140 μ L plasma RNA coupled to Carrier RNA in Buffer AVL and 96-100% ethanol was centrifuged and bound to a filter membrane. 60 μ L RNA was eluted with Buffer AW1 and AW2 through a spin column. All reagents except ethanol supplied by kit.

Reverse-transcriptase PCR: 10 μ L RNA was then reverse transcribed into cDNA, in duplicate, with the reaction mixture (20 μ L): 2.9 μ L RNase/DNase-free water (Promega); 3 μ L 10 \times Taqman buffer A (Applied Biosystems); 6 μ L MgCl₂ (25nM) (Applied Biosystems); 1.5 μ L Randon Hexamers (diluted 1/2; Applied Biosystems); 6 μ L dNTPs (2.5nM; Promega); 1.5 μ L; Promega); 0.5 μ L Rnasin (40U/mL; Promega); 0.1 μ L MMLV-RT superscript (200U/mL; Invitrogen), for one thermal cycle: 25° C (15min) \rightarrow 42° C (40min) \rightarrow 75° C (5min) (GeneAmp PCR System 9700, Applied Biosystems). A third test per sample was set up to assess the presence of SHIV DNA contamination, using the same reaction mix excluding MMLV-RT superscript. SIV RNA standards (33) were serially diluted and reverse-transcribed in duplicate (limit of detection, 1500 copies/mL).

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Real-time PCR: cDNA was amplified with reaction mixture (20 µl): 141µl RNase/DNase-free water (Promega); 2µL 10× PCR buffer II (Applied Biosystems); 1 µL MgCl₂ (Applied Biosystems); 1 µL SL03 SIVgag (20pmol/µL); 1µL SL04 SIVgag (20 pmol/□L); 0.3 µL SL07 molecular beacon 0.5µL Tag Gold (Applied Biosystems) as
5 previously described (33). Reaction temperature was initially raised and held at 95 °C for 10 min to activate Tag Gold enzyme, followed by 45 thermal cycles: 95° C (15 sec) → 55° C (30 sec) → 72 °C (30 sec). Real-time analysis was performed on amplicon detection at 55° C (30 sec) stages by Sequence Detector software v1.6.3 (Applied Biosystems).

CD4+ T cell counts

10 To assess the depletion of CD4+ T cells following SHIV challenge, 200 µL whole blood was incubated with 5 µL PE-conjugated anti-human CD3, 5 µL FITC-conjugated anti-human CD4, 5 µL PerCP-conjugated anti-human CD8 (clone SP34; L200, and; Leu-2a, respectively; BD Pharmingen) monoclonal antibodies for 20 min in dark, RT. Red
15 blood cells were lysed with 2 ml FACS Lysing Solution (BD Biosciences) and fixed as described in method 2.8. 50,000 total events were collected by 3-colour FACScan Calibre® and CD4+ and CD8+ T cell counts expressed as the percentage of gated lymphocytes.

RESULTS

In vivo cytotoxic T-lymphocyte killing

20 The standard measure of virus-specific CTL effector is measured *via* the release of a radioisotope ⁵¹Cr from target cells, an assay that is tedious and poorly sensitive. By pulsing dye-labelled autologous macaque PBMC with large pools of SIV and SHIV overlapping peptides (OPAL) and infusing the cells back into the same animal, the
25 inventors were able to kinetically show SHIV-specific killing in blood sampled at various time-points following the infusion of OPAL by flow cytometry.

Two weeks after full immunisation (week 10), three of four immunised animals displayed moderate to large (11.4-76%) killing of gag-pulsed PBMC by 16 hours post-OPAL infusion, whereas control-immunised monkeys displayed <7% gag-specific killing. One immunised animal, monkey H20, demonstrated vigorous gag-specific killing (27.3%)
30 as early as 4 hours post-infusion (Figure 6). These data were consistent with T cell responses induced by the vaccines as analysed by IFNγ ELISpot and ICS (data not shown),

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indicating the usefulness of OPAL to measure effective CTL effector responses primed by the DNA and FPV vaccines.

Shortly (2 weeks) after SHIV intrarectal challenge all four immunised animals exhibited large degrees of gag-specific killing (65-98.3%) 16 hours post-OPAL infusion, and two of four (monkeys H20 and H21) further demonstrated >99% pol-specific killing (Figure 7). In comparison with control-immunised animals, monkey E20 displayed <6% killing of both gag- and pol-pulsed PBMC whereas monkey E22 showed >90% and 31.9% of gag- and pol-pulsed PBMC, respectively. Interestingly, the animals that displayed moderate to high degrees of pol-specific killing (monkeys H20, H21 and E22) were also the only animals that had previously received 2 doses of infused pol-pulsed PBMC (weeks 10 and 15), whereas monkeys B00, H8 and E20 received pol-pulsed PBMC only once prior. This observation suggests that the infusion of OPAL may have: (a) boosted pol-specific T cell responses primed by the vaccines that were weakly or not detected by IFN γ ELISpot and ICS (data not shown), and; (b) induced pol-specific immunity in naïve animals evident post-SHIV challenge.

Analysis of the immunogenicity induced by infusing peptide-pulsed autologous cells.

It seemed plausible that if *in vivo* CTL killing could be efficiently measured by OPAL infusion, this method may be able to either prime a new or boost an existing immune response. IFN γ ELISpot and ICS assays were therefore performed prior to- and one week following each OPAL infusion assay to analyse whether there would be an increase in T cell immunogenicity previously primed by the vaccines or by the OPAL infusion method itself (Figure 8).

Following the first OPAL infusion performed at week 10, a 3- to 16-fold increase in IFN γ -secreting cells to SIV gag peptide pool was detected in monkeys H20 and H21, measuring up to 430 spot-forming cells (Figure 9). Monkey H8 measured a 54% increase to 215 spot-forming cells, whereas no increase was measured in control-immunised animals. Analysis of monkeys B00 (post-OPAL infusion) and E20 (pre-OPAL infusion) for all antigens analysed were excluded due to developmental problems of the assay. Of the four animals that received pol-pulsing at week 10, monkeys H20, H21 and E22, displayed increased pol responses by up to 140 spot-forming cells post-OPAL infusion, whereas no significant ELISpot responses were detected in monkey E20. No nef-specific T cell was in all animals apparent before or after OPAL-infusion. These results suggest a

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boosting effect in T cell immunogenicity following gag- and pol-peptide pulsing in the animals previously primed for SIVgag/pol responses, and furthermore indicate priming for SIVpol in a naïve animal (monkey E22).

At week 15, 8 weeks following full immunisation, a second OPAL infusion assay was performed in the six animals. ELISpot analyses revealed increased responses to gag peptide pool by up to 500 spot-forming cells from approximately 50 or less spot-forming cells prior to OPAL infusion in the four animals pre-immunised with DNA and FPV vaccines. In control-immunised animals, no gag-specific T cells were measured before or after the assay (Figure 10). In comparison, a slight increase in pol-specific responses (up to 40 spot-forming cells) from baseline was measured in only a few animals. Large increased responses to WI SIV were measured in all pre-immunised animals (up to 450 spot-forming cells), whereas control-immunised animals displayed modest or no increases (up to 50 spot-forming cells). All responses to SIV nef and SHIV env were minimal or undetected in all animals prior to and after OPAL infusion.

Following SHIV intrarectal challenge, all animals except monkey E20 displayed increased gag responses measuring between 50-600 spot-forming cells. Similar responses were observed for WI SIV but to levels up to 200 spot-forming cells, whereas pol responses above 50 spot-forming cells were only evident in monkey H20.

The immunogenicity of OPAL infusion was further verified by comparison to animals that received the same immunisation regimen but did not receive OPAL infusion (Figure 11). No rise in SIV gag, pol or WI SIV-specific T cells were detected in groups 1 (control-immunised) and 2 (2×DNA/FPV-immunised) from weeks 9 to 11 and 15 to 18. Responses from weeks 20 to 21 increased slightly the groups, attributable to responses enhanced by SHIV challenge at week 18.

25 Outcome of SHIV_{mn229} intrarectal challenge

The highly pathogenic SHIV_{mn229} challenge stock was inoculated intrarectally into all macaques 10 weeks after full immunisation at a dose of 10^5 TCID₅₀. Plasma SHIV RNA and CD4⁺ T cell counts were followed in all control-and 2×DNA/FPV-immunised animals (Figure 12).

30 Control-immunised monkeys E20 and E22 exhibited peak viral loads of 7.8 ± 0.7 log₁₀ copies/ml at 2 weeks following challenge. The peak viral load of monkey E20 may

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have occurred between week 1 and 2, however, set-point levels of both monkeys (measured 5 to 11 weeks post challenge) remained high at $5.9 \pm 0.3 \log_{10}$ copies/mL. Conversely at week 2, CD4+ T cell counts dropped dramatically to $1.6 \pm 1.1\%$ of total lymphocytes, and set-point levels were steady at $0.3 \pm 0.2\%$. Monkeys that received the same immunisations but no OPAL infusions (group 1) performed only marginally worse than monkeys E20 and E22 in terms of peak and set-point viral loads ($8.2 \pm 0.1 \log_{10}$ copies/ml and $6.2 \pm 0.3 \log_{10}$ copies/ml), as well as CD4+ counts (set-point $0.5 \pm 0.3\%$).

Based on the enhanced pol-specific killing that may have been attributed to 2 separate OPAL infusions, the SHIV viral loads and CD4+ T cell counts of monkeys H20 and H21 were compared to monkeys B00 and H8 that received only 1 dose of pol-OPAL infusions. Peak viral load of monkeys H20 and H21 (receiving 2 pol-OPAL infusions) was at least 10-fold lower than monkeys B00 and H8 (5.9 ± 1.3 vs. $7.1 \pm 0.4 \log_{10}$ copies/mL, $P=0.08$), and set-point viral load showed a trend towards being lower (4.1 ± 0.9 vs. $5.4 \pm 0.7 \log_{10}$ copies/ml, $P=0.08$, student's *t* test). Incidentally, set-point CD4+ T cell count for monkeys H20 and H21 was significantly greater than monkeys B00 and H8 ($18.9 \pm 6.1\%$ vs. 8.4% , $P=0.02$). Although statistically insignificant in comparison with group 2 animals who received the same immunisations but no OPAL infusions ($P=0.12$), monkeys H20 and H21 that received multiple pol-OPAL infusions displayed a trend towards the retainment of CD4+ T cells although viral loads were relatively similar, indicative of viral challenge protection. Set-point CD4+ T cell count and viral load of group 2 were $13.0 \pm 3.7\%$ and $4.8 \pm 0.2 \log_{10}$ copies/ml, respectively.

In comparison to control-immunised monkeys E20 and E22, both set-point viral load and CD4+ T cell count of monkeys H20 and H21 were significantly different ($P=0.01$, $P=0.00$). The set-point viral load of monkeys B00 and H8, on the other hand, was not significantly lower than monkeys E20 and E22 ($P=0.37$) despite significant set-point levels of CD4+ T cells ($P=0.01$). Note that monkey H20 had completely cleared plasma viral RNA from week 5 and onwards and retained CD4+ T cells at normal levels.

DISCUSSION

The vital role for HIV-1-specific CD4+ T-helper (Th) and CD8+ CTL responses in controlling HIV-1 replication is the focus of many current vaccine concepts. The infusion of autologous PBMC pulsed with large overlapping sets of SHIV 15mer peptides (OPAL) was surprisingly immunogenic in its ability to boost SHIV-specific immune

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responses as analysed by IFN γ ELISpot and ICS assays. This finding forms the potential basis of a novel vaccine or immunotherapeutic strategy as described herein.

The evidence for this immunogenicity was five-fold: (a) Increases in SIV gag-specific IFN γ ELISpot responses were observed one week after each of the 3 SIV gag OPAL infusions (week 10, 15, and 20) in all vaccinated monkeys. In contrast, at week 10 and 15, SIVgag responses in equivalently immunised animals (group 2) not receiving the OPAL infusion significantly declined. (b) Increases in SIV pol-specific IFN γ ELISpot responses were observed in immunised animals one week following the SIV pol infusion at week 10 and 20. Interestingly this was observed in only the two monkeys H20 and H21 that received multiple SIV pol OPAL infusions prior to SHIV challenge (weeks 10 and 15) and not in animals receiving SIV pol peptide pulsed cells at week 15. This is of particular interest since the pol-specific T cell responses to the DNA and FPV vaccines alone were modest or undetectable by ELISpot and ICS. (c) High levels of SIV pol-specific *in vivo* killing were also seen in the two monkeys that received 2 prior infusions of SIV pol OPAL infusions. (d) This immunogenicity data was further confirmed by high levels of SIV pol-specific IFN γ intracellular cytokine responses in the two immunised animals receiving the multiple SIV pol OPAL infusions. (e) There was a trend towards greater protection from SHIV challenge in animals receiving multiple OPAL infusions. Together, these results suggest that pulsing autologous PBMC *ex vivo* with pools of overlapping peptides is an effective method for boosting immune responses.

There is a body of data ascertaining the use of pulsing autologous or syngeneic cells with defined peptide epitopes or whole antigen for the induction (or 'cross-priming') of immune responses (22, 23, 27, 34, 35). The use of specialised antigen presenting cells such as monocyte-derived dendritic cells pulsed with, for example, single tumour antigens or whole inactivated SIV has also been studied extensively as an immunotherapeutic tool (36-39). However, to the inventors' knowledge this is the first report of utilising large peptide pools spanning an entire protein (125 SIV gag 15mers or 263 SIV pol 15mers) and the use of whole PBMC cultured for short periods *ex vivo*, as a method of boosting immune responses.

In one control-immunised animals, monkey E22, which received multiple infusions of SIV pol (and SIV gag) pulsed cells, a modest induction of SIV gag and SIV pol-specific IFN γ ELISpot responses was detected. This animal subsequently had high

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levels of SIV gag- and pol-specific killing analysed at week 20, presumably from the boosting effect of the SHIV challenge. The efficiency of priming an immune response by OPAL infusion therefore seems feasible.

Further modifications to the OPAL technique, such as the enrichment for APC
5 and/or dendritic cells (DC) (40), would potentially enhance the immunogenicity of OPAL
infusion as a therapeutic vaccine since DC cultured from PBMC of HIV-infected patients
(41, 42) and SIV-infected animals (40) can elicit potent T-cell responses. Alternatively, the
prospect of using whole blood rather than PBMC fractions as a means of delivering OPAL
will certainly benefit a clinical setting, particularly for HIV-infected persons. Furthermore,
10 a smaller whole blood sample may not require as high a concentration of peptide since
1 µg/mL is effective *in vitro* for whole blood analysis by ICS. It is also conceivable that
direct intravenous infection of pooled peptides would mimic the immunogenicity of the
OPAL effect. The use of consensus HIV-1 clade peptide sets of gag and pol offers the
broad epitopic breadth desired of an effective therapeutic vaccine for humans. The
15 immunogenicity of antigens that regulate viral replication, such as rev, tat, vpu, vif and
vpr, which are poorly immunogenic by current vaccine approaches, should also be
improved using this strategy.

The disclosure of every patent, patent application, and publication cited herein is
hereby incorporated herein by reference in its entirety.

20 The citation of any reference herein should not be construed as an admission that
such reference is available as "Prior Art" to the instant application.

Those skilled in the art will appreciate that the invention described herein is
susceptible to variations and modifications other than those specifically described. It is to
be understood that the invention includes all such variations and modifications. The
25 invention also includes all of the steps, features, compositions and compounds referred to
or indicated in this specification, individually or collectively, and any and all combinations
of any two or more of said steps or features.

DATED this 10 June, 2003

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MYRNSVLSGKKADELEKIRLRPNCKKYNLKVWAAEIDRFGLASLLENKBCQKLSVLAPVPTSENLASLNTVCVNCIHAEKVHTESAQIVORHLVETGTTTPTCTSPFAPSRCGGNYPVQIQGNYVHLPSPTLANVVLTEKKFGLEVV
 (1) MGVRSVLSCKKDE
 NSVLSGKKADELEK
 SQUADELEKIRLP
 ADELEKIRLPNCK
 EKIRLPNCKKYNL
 LRPNCKKYNLKVW
 GKCKNKLKVVWAAE
 YLKHVWAAEIDRF
 HWAAEIDRFGLA
 (10) AAEIDRFGLASL
 LORGLASLLENK
 GLASLLENKBCQK
 SLENKBCQKLSV
 NKEGCKLSVLAPL
 QKLSVLAPLPTG
 LSVLAPLPTGSENL
 PUSENLGLNTVC
 ENLSLNTVCVNC
 (20) SLNTVCVNCIHAE
 IMCHAEKVHTESAQI
 HABEKVHTESAQI
 KVHTESAQIVORH
 TEAKQIVORHLVVE
 KQIVORHLVETGTT
 ORHLVETGTTTPT
 VETGTTTPTCTSP
 GTTPTCTSPFAPS
 (30) TPTCTSPFAPSSG
 TSPFAPSSGCGNY
 TAPSSGCGNYPVQ
 SGCGNYPVQIQG
 GNYVQIQGNYVHL
 VQIQGNYVHLPLP
 GNYVHLPLSPPTLN
 VHLPLSPPTLANV
 LSPPTLANVVLTE
 TLANVVLTEKKFG
 (40) NVLTEKKFGLEVV
 IEEKFGASVVPFQALSECTPYDINQVLCVGDHQAQOIIRDIINEEAUMLQHPAPPOCQOLREFSGDLAGTSSVDEQIQMVNQCPPIPVGNITERNIQLQKCVRNWPTNILLVKGPEKPPQSYDREYKSLRSEQTDAAVENHATQTLITONANPDC
 (41) IEEKFGASVVPFQ
 KFGASVVPFQALSE
 ENTEGQALSECTP
 GQALSECTPYDIN
 LSECTPYDINQVLC
 CTPYDINQVLCVGD
 DINQVLCVGDHQA
 MLCVGDHQAQOII
 VGDHQAQOIIIRDI
 (50) QAMQOIIIRDIINEEA
 QIIRDIINEEAUML
 DIINEEAUMLQHP
 HEADMLQHPQAP
 DMLQHPQAPQOQ
 QHPQAPQOQOLRP
 PAPQOQOLRPSGD
 CQOLRPSGDLAGT
 RPSGDLAGTSSV
 GSDLAGTSSVDEQI
 (60) AGTSSVDEQIQMV
 ACVNTNPTNILLV
 (70) MNTNPTNILLVKG
 DVKQKPEKPPQSYD
 GPEKPPQSYDREYK
 PPSYDREYKSLR
 YDREYKSLRSEQTD
 FYSLRSEQTDAAV
 LPSQTDAAVENHAT
 QTDAAVENHATQTL
 AVENHATQTLITON
 (80) NKTQTLITONANPDC

FIGURE 1

TLIIQIANPCKLVKGLGNPTLEEMHTACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 (161) TLIIQIANPCKLVKGLGNPTLEEMHTACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 QNAMPCKLVKGLGNPTLEEMHTACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 PCKLVKGLGNPTLEEMHTACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 LVLKGLGNPTLEEMHTACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 GLGNPTLEEMHTACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 EEMHTACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 TACQGVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 GVGGGQOARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 (90) PGQARLMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 ARUAMAEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 AEALKEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 KEALAPVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 APVTFPAAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 IPFMAAGQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 AQOQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 RQBSPRKPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 KPIKCNQKECHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 CNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 (100) GCHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 (101) HSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 QCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 PRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 GCHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 GCHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 DRUMACPDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 AKCPDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 DRQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 GFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 (110) LSPKCKKPRPMAQ
 GCHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 RPFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 MQVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 HQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP
 MPDPAVLLKRYNQLZHQOKEQKRESKTP
 PPDPAVLLKRYNQLZHQOKEQKRESKTP
 PAVLLKRYNQLZHQOKEQKRESKTP
 LKRYNQLZHQOKEQKRESKTP
 YNQLZHQOKEQKRESKTP
 (120) GCHSARQCRAPRQGCNHCQGHVMAKCYDQAGFLGLAFHGGKCPENFMAOVHQGLMFTAPFSDPAYDILLKRYNQLZHQOKEQKRESKTP

REKQRESKTPKYEVTEDLHNSLFGSDQ
 (121) REKQRESKTPKYEVTEDLHNSLFGSDQ
 RESKTPKYEVTEDLHNSLFGSDQ
 EKPYEVTEDLHNSLFGSDQ
 KEVTEDLHNSLFGSDQ
 (125) EDLHNSLFGSDQ

FIGURE 1 contd

[illegible]

FIGURE 2

DAYFSIPLDEEPROTATLPSVWNAEPGEVIEVLPOCKMSPAIFQVTHRVLEZPPRANPDVTLQVHODDILLASDRTDLEHDKVVLQSTELLANSIGFSTPEKQKDPFPQMGYELAPTKKLOKIELPQRETNVNDIQKLVGLVWAAQIYFGIKTRHL
 (81) DAYFSIPLDEEPROT
 SLPDEEPROTAT
 DEEPROTATLESV
 ROTATLPSVWNAE
 ATLPSVWNAEPKRR
 PSVWNAEPKRRYIK
 KAPKRRYIKVLPQ
 GERIYNKLPQKNG
 IYKVLPOCKMSPAI
 (90) LPOCKMSPAIFQV
 WKSSPAIFQVTHRV
 PAIFQVTHRVLEP
 QVTHRVLEPFRKAN
 RVLEPFRKANPDVT
 EPPFRKANPDVTLQV
 KAPDVTILQVRODI
 DVTILQVRODILLAS
 VQVRODILLASDRTD
 ODILLASDRTDLEH
 (100) IASDRTDLEHDRVUL
 (101) RTDLEHDRVULQSK
 BHDVULQSKELLANS
 VULQSKELLANSIGFS
 SELLANSIGFSTPE
 LMSIGFSTPEKRR
 GFSSTPEKRRQPPF
 PEEKRRQPPFQNG
 FQKPPFQNGYELM
 PPFQNGYELAPTKM
 (110) WNGYELAPTKKLOK
 ELMPKTKKLOKIELP
 TKKLOKIELPQRET
 LQKIELPQRETIVH
 ELPQRETIVNDIOK
 RETIVNDIOKLVG
 TVNDIOKLVGLVWNA
 IQKLVGLVWNAQIY
 VGLVWNAQIYFGIK
 WNAQIYFGIKTRHL
 (120) QIYFGIKTRHL
 GIATKHLCHLJRGIMTEEVQNTMNAEYBENKIILSQEGCTYQSGXPLBATVINSQONQSVNKHQBCKILANGKFAKIGTHNGVLLANVIOKIGKBAIVINGQVPRFLPVEKOVHQBOWTDVQVTHIPENDFISTPFLVFLVFLVNDPDEGETTYTDDG
 (121) GIATKHLCHLJRGIM
 KHLCHLJRGIMTE
 RLJRGIMTEEVQNT
 GIMTEEVQNTMA
 LTESVQNTMAEY
 VQNTMAEYBENK
 ENAEYBENKILAS
 AYEYBENKILSQOR
 ENKILSQORGCY
 (130) ILSQORGCYQSG
 EQSGCTYQSGPBA
 CYQSGCTYQSGPBA
 EGPBAATVINSQON
 LBATVINSQONQSV
 VINSQONQSVKIHQ
 QSVKIHQSGZKI
 ASYKIHQSGZKILNG
 IHQSGZKILNGKFAK
 DKILNGKFAKIRNT
 (140) KVGKFAKIRNTNG
 KVGKFAKIRNTNG
 TNGVLLANVIOKIG
 RLJRGIMTEEVQNT
 HVLQKIGKBAIVING
 KIGKBAIVINGQVPR
 EAVINGQVPRFLP
 IAVINGQVPRFLPVEK
 VPRFLPVEKOVHQB
 (150) HLFVEKOVHQBOWTD
 SEDVVEQOWTDYQV
 WQOWTDYQVTHIP
 WQOWTDYQVTHIPEND
 WQVTHIPENDFISTP
 WPFISTPFLVFLV
 WPFISTPFLVFLVFLV
 STPFLVFLVFLVFLV
 IAVLFLVFLVFLVFLV
 VFLVFLVFLVFLVFLV
 (160) VFLVFLVFLVFLVFLV

FIGURE 2 contd

(161) IEGSEYTYDGSCHQSGGKAGYITDREKGDVNVLEQTTNQDALEKAPVALLTDSGPKANIIVDSQYVWGOITTCQPTTSESSLVNOIIEBNIKESEIYVAVPFAHKIGCGNOEIDHVLVSOQITRQVLTLEKTEPAQDEEDHVKYSHVKELYVPEFGLPRIVARQIVDTQCDKNC
 EYTYDGSCHQSGKE
 TYTYDGSCHQSGKE
 TDSCHQSGKEGKAG
 CNQSGKEGAGYITD
 SEEGKAGYITDRGKD
 KAGYITDRGDVNV
 ITDRGEDVNVLEDT
 GDNVNVLEDTNQO
 VYVLEDTNQDALE
 (170) EOTTNQDALEKFLM
 NOOLEKFLMALT
 ELEKFLMALTDSGPK
 FLAVLTDGPKANI
 LTDSGPKANIIVDSQ
 GPKANIIVDSQYNG
 NIIVDSQYNGITTC
 DSQYNGITTCQPTT
 VMGIITTCQPTTBSR
 ITTCQPTTBSSELVNO
 (180) PTSESSLVNOIIEK
 (181) ESRVNOIIEBNIKE
 VNOIIEBNIKESEIY
 IEMIKSEIYVAV
 IKKSEIYVAVPFAHK
 EIVVAVPFAHKIGG
 AVVPFAHKIGCGNOE
 AHKIGCGNOEIDHVL
 ICGNOEIDHVLVSOQIT
 QEIDHVLVSOQITRQVLT
 (190) HFSQSGITRQVLTLEK
 QGIRQVLTLEKLEKPA
 QVLAFLAKLEPAQEEH
 LEKLEPAQEEHRYH
 EPAQDEEDHVKYSHVK
 EEDHVKYSHVKELVLA
 KHYSHVKELVLAFFGL
 BVKELVLAFFGLPRIV
 LVFAFFGLPRIVARQIV
 FGLPRIVARQIVDTQCDK
 (200) RIVARQIVDTQCDKNC

[illegible]

FIGURE 2 contd

(241) MTPAERLINIITTEQEIQFOQSKNSKFRPNVYTPREGRDQWKGPCELLWNGEAVILKVGTDIKVVPREKAKIIKDYGGKGVDSSSHMEDTGEAREVA
 ENLINIITTEQEIQF
 NM1ITTEQEIQFOQSK
 TEQEIQFOQSKNSK
 IQFOQSKNSKFNFR
 QSKNSKFNFRVYR
 SKFNFRVYRGRD
 NFRVYRGRDQWAK
 YPREGRDQWKGGE
 (250) GRDQWKGPEELLAK
 LKNGPOLLWNGE
 PELLWNGEAVILK
 LKNGEAVILKVGTD
 BGAVILKVGTDIKV
 ILKVGTDIKVVPREK
 GTDILKVPREKAKII
 KVPREKAKIIDYG
 RRKAKIIDYGGKGE
 KIIDYGGKGEVDSS
 (260) DYGGKGEVDSSHME
 (261) GKEVDSSSHMEDTGEAREVA
 DSSSHMEDTGEAREV
 (263) HMDTGEAREVA

FIGURE 2 contd

(1) MGAISHERSPGDLQRIIPARGETVGRLLGEVBDGVSQSPGDLKGLSLSCEGKYNQYMTPHRPAERREKLAVERKQWDDID
 (21) ATRKQWDDID
 ISMRSPGDLQRI
 NRPSPGDLQRI
 SGLQRIIPARGET
 RQRIIPARGET
 LPARGETVGRLLGEV
 GETVGRLLGEVBDGVSQSP
 GRLLGEVBDGVSQSP
 GEVBDGVSQSPGLD
 (10) LCTSPGDLKGLS
 QSPGDLKGLSLS
 GLDGLSLSCEGKYNQYMT
 GLSLSCEGKYNQYMT
 LSCEGKYNQYMT
 CQVBDGVSQSP
 NCGVBDGVSQSP
 YMTPHRPAERREK
 PHRPAERREKLAVER
 PAERREKLAVERKQW
 (20) REKLAVERKQWDDID

FIGURE 3

PRVAGI RRTYQELARGOTLLGMLHICSAVEXLNVTVYGVPAKREATTTLFCASDAKYDTEVHVHATHACVPTDHPQEIENVTEFRHNNVEQHHEDIISLMDQSLEPCVGLTFLACTHCTHLEHATNTSSNNKKNRGEIXKCSFNVTTISIGNDQKE
 (1) PRVAGI RRTYQELARGOTLL
 GIKRKYQELARGOTLL
 NYOHLARGOTLLG
 LNRGOTLLGMLHIC
 GTIAGMLHICSAVE
 LGMHICSAVERLW
 HICSAVERLNVTVY
 AVERLNVTVYGVPA
 LNVTVYGVPAKRE
 (10) VYGVPAKREATTTL
 VPAKREATTTLFCAS
 KEATTTLFCASDAKA
 TTLFCASDAKAYOTE
 CASDAKAYOTEHNV
 AKAYOTEHNVHATH
 DTEVHVHATHACVPT
 HVMHATHACVPTDEN
 ATHACVPTDENHQEI
 CVPTDENHQEIVLEN
 (20) DENHQEIVLENTEN
 VLENTEFRHNNVEQ
 FENHNNVEQHHEDIISL
 KNNVEQHHEDIISL
 VEOHHEDIISLMDQS
 HEDIISLMDQSLEPC
 ISLMDQSLEPCVGLT
 DASLEPCVGLTFLCTV
 (30) EPCVGLTFLCTVHIC
 KLPFLCTVHICTNL
 LCVHICTNLHLEHATNTSS
 NLEHATNTSSNNKKNRGE
 ATNTSSNNKKNRGEIX
 TSSNNKKNRGEIXKCSF
 WKNNRGEIXKCSFNVTTIS
 NGEIXKCSFNVTTISIGN
 (40) SPVTTISIGNDQKE

TTBICHRMKEYALFYELDVPIDNDNTSYNLINCVTSVITQACPVNEFPIHYCAPAGPAILKNDKKNKSGPCINVTQCTHGLRPMVSTOLLASABQWIRBENFTDNVKTIIVQLKESVEINCTRPENHTRPSIPYQPKAFYATGDIIGDIHQACH
 (41) TTBICHRMKEYALFYELD
 GURMKEYALFYELD
 HQKEYALFYELDVP
 YALFYELDVPIDND
 YELDVPIDNDNTSY
 VVPIDNDNTSYLILB
 DNDNTSYNLINCVTS
 TSYNLINCVTSVITO
 LINCNTSVITQACPK
 (50) NTSVITQACPVNEF
 ITOACPVNEFPIHY
 CPKVSEFPIHYCA
 SFEPPIHYCAPAG
 IPHYCAPAGFALK
 YCAPAGFALKNDK
 AGFALKNDKKNKSG
 LKNDKKNKSGPCIN
 NDKKNKSGPCINVS
 FNGSGPCINVTQCT
 (60) CPCINVTQCTHGL
 VQCTHGLRPMVSTOLL
 HGLRPMVSTOLLASAB
 FVSTOLLASABQWIR
 TOLLASABQWIRBEN
 LMSLABQWIRBENFT
 LAEROWIRBENFTDN
 GUVIRBENFTDNVKT
 RSEFTDNVKTIIVQL
 (70) FTRVKTIIVQLAS
 VKTIIVQLASVEIN
 IVQLASVEINCTRP
 KESVEINCTRPENH
 EINCTRPENHTRPSI
 TRPNHTRPSIPYQPK
 HTRPSIPYQPKAFYAT
 KSPYQPKAFYATGDIIG
 KAFYATGDIIGDIHQACH
 (80) ANGDIIIGDIHQACH

FIGURE 4

[illegible]

(121.) YXVKLEPFGVFTTAKRVRQREKAVTIGAVTGFGLRAGSTGMAEILTVQARQLLSGIVQOQWLLRA TEAQORLQJLTVWGHGKQARVLAVERYLQOQJLGLGCSGELICTTANFNAASHNSKSLQJQNNNTWMEEREICGYNLNLITVLIESQOQJQ
YXVKLEPFGVFTTK
KIEPLGAVPTK
LGAVPTKRAER
FTKRAERVQREKRA
KERVQREKRAVTLG
VQREKRAVTLGAVFL
KRAVTLGAVTLGFLG
TLGAVTLGFLGAGS
VELGFLGAGSTMA
(130) FZGAGSTMAEILTVQ
AGSTMAEILTVQ
HGAASLITVQARQL
SLITVQARQLLSGI
TVQARQLLSGIVQOQ
QLLSGIVQOQWLL
SELVQOQWLLRAE
VQOQWLLRAEADQ*

(140) AQOQLLQTVMGIKQ
RAIENQOQLLQTVW
NLLRAEADQOQLLQ
VQOQWLLRAEADQ*

(150) ELIYVLIESQOQJ
GATNLITVLIESQ
BREIGNITNLITVL
WMEEREICGYNLNL
NMTWMEEREICGYN
DOINMTWMEERE
HESLQJQNNNTWME
ASPSKSLQJQNNNT
VPMNAASHNSKSLQ
CTTAVPMNAASHNS
(150) GELICTTAVPM
HCSGELICTTAVPM
LHGIMCSGELICTT
KQOQLLQJHCSGEL
ERVLQOQLLSGIC
ERVLQOQLLSGIC
VLAVERTLQOQLLG
LQARVLAVERTLQO
LQARVLAVERTL
LQOARVLAVERTL
TVGHGKQARVLA
TVGHGKQOQJLQ

(141) LQJLTVWGHGKQOQJ
TEAQORLQJLTVWGHGKQARVLAVERYLQOQJLGLGCSGELICTTANFNAASHNSKSLQJQNNNTWMEEREICGYNLNLITVLIESQOQJQ

FIGURE 4 contd

NIQELKNSAVSFGAIALNAVSGTDRIEVAQRIGCNFCHIPPRIRQGLERTLL
 (201) NIQELKNSAVSFGAI
 LKNSAVSFGAI
 NIQELKNSAVSFGAI
 AVSFGAIALNAVSG
 FGAIALNAVSGTDRI
 ALNAVSGTDRIEVA
 ASGTDRILEVAQRIG
 DRIEVAQRIGCNFCH
 EVAQRIGCNFCHIPPR
 RIGCNFCHIPPRIRQ
 (210) AFCHIPPRIRQGLER
 (211) IPPRIRQGLERTLL

FIGURE 4 contd

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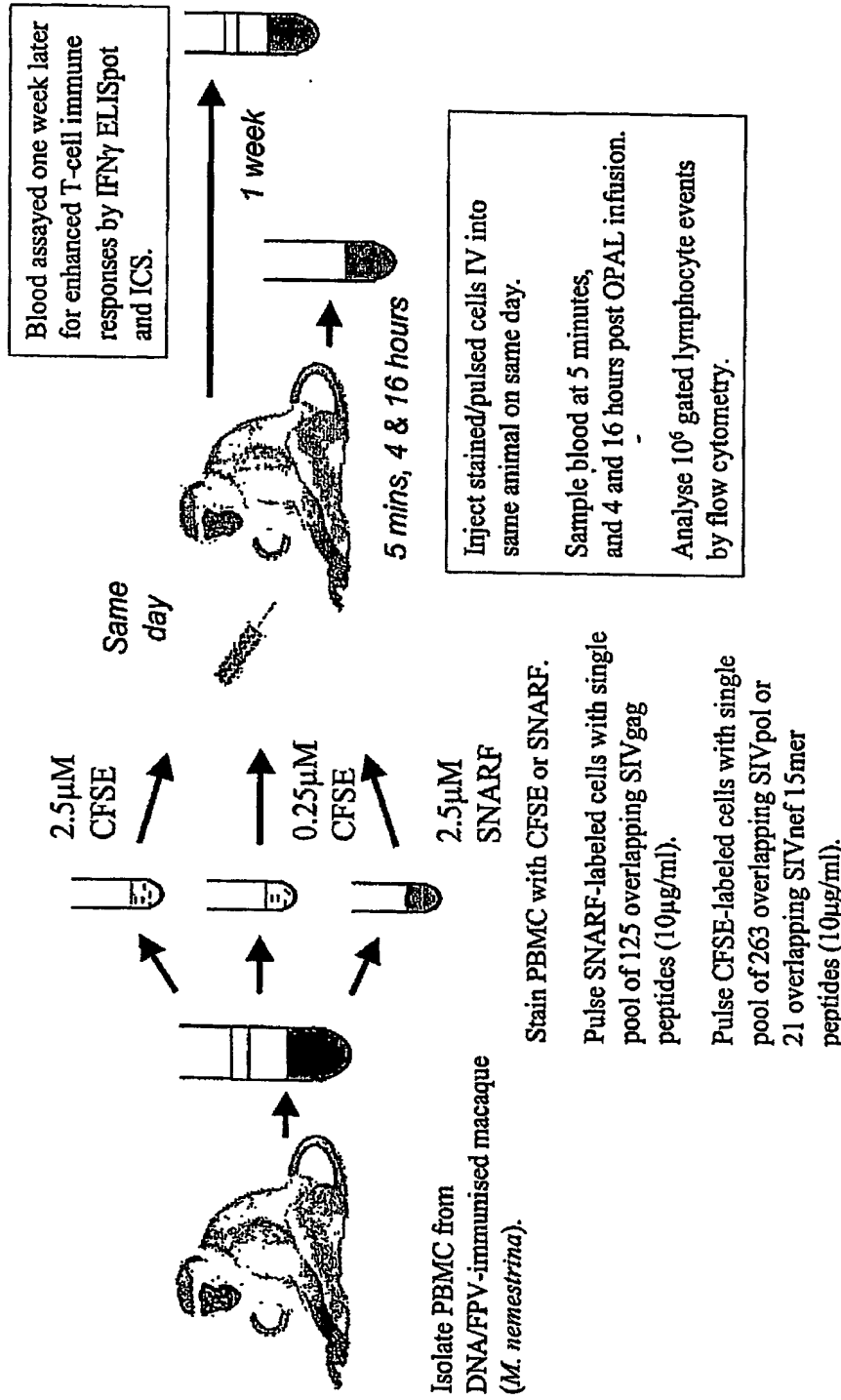


FIGURE 5

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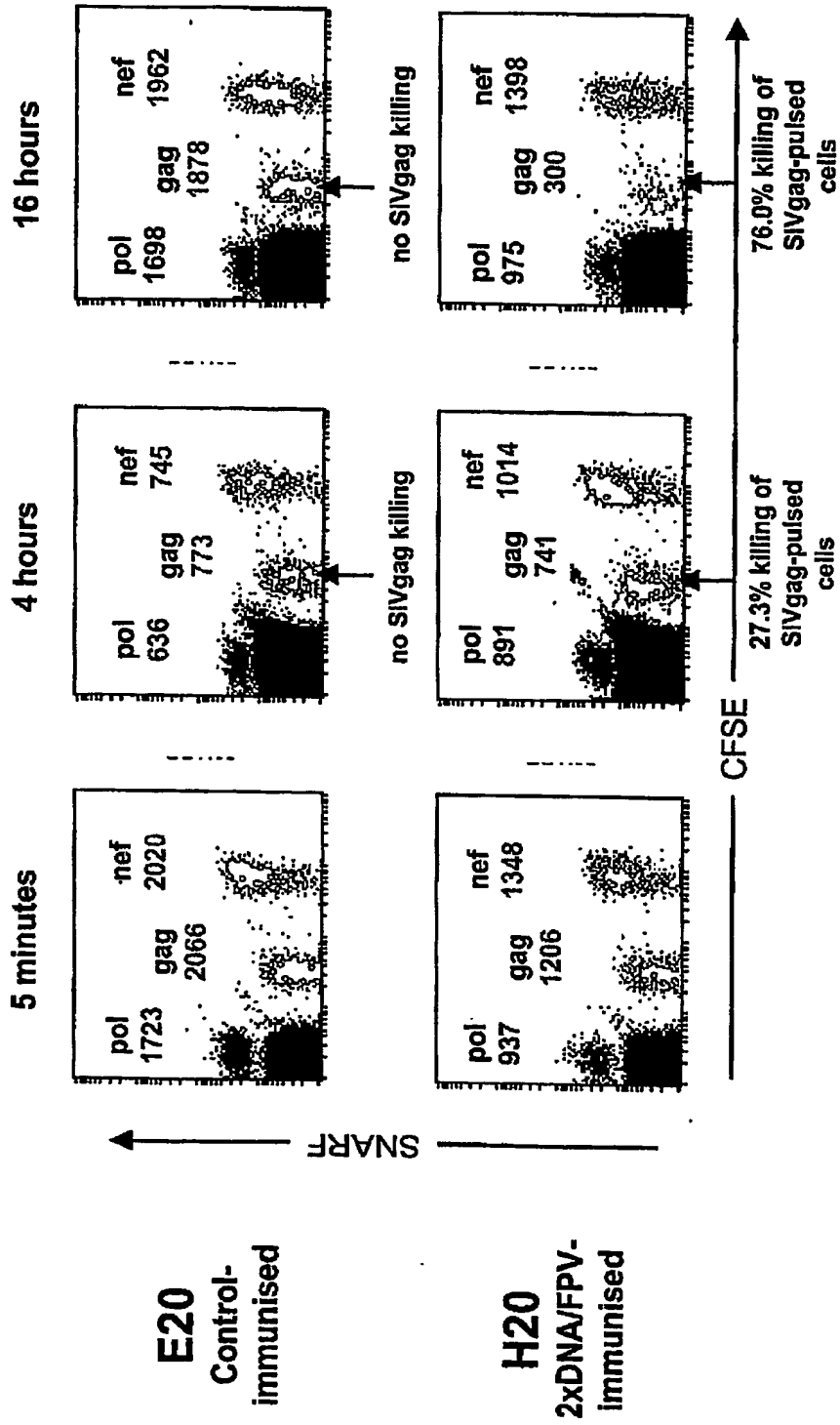


FIGURE 6

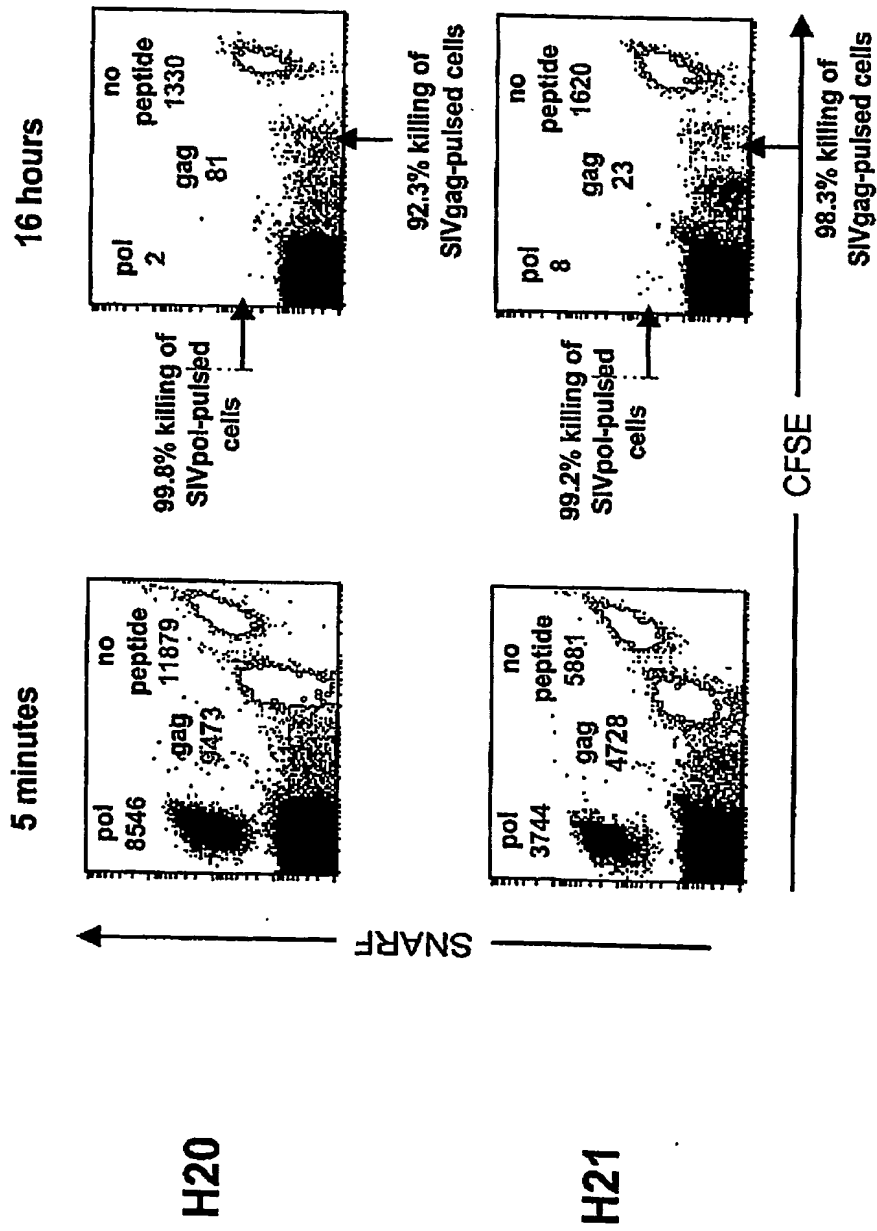
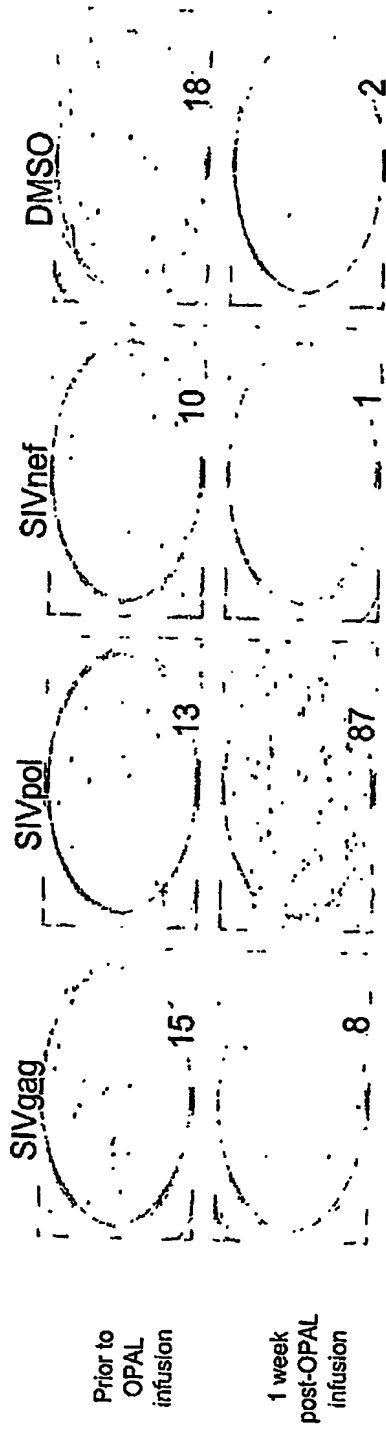


FIGURE 7

E22 - control-immunised animal



H20 - 2xDNA/FPV-immunised animal

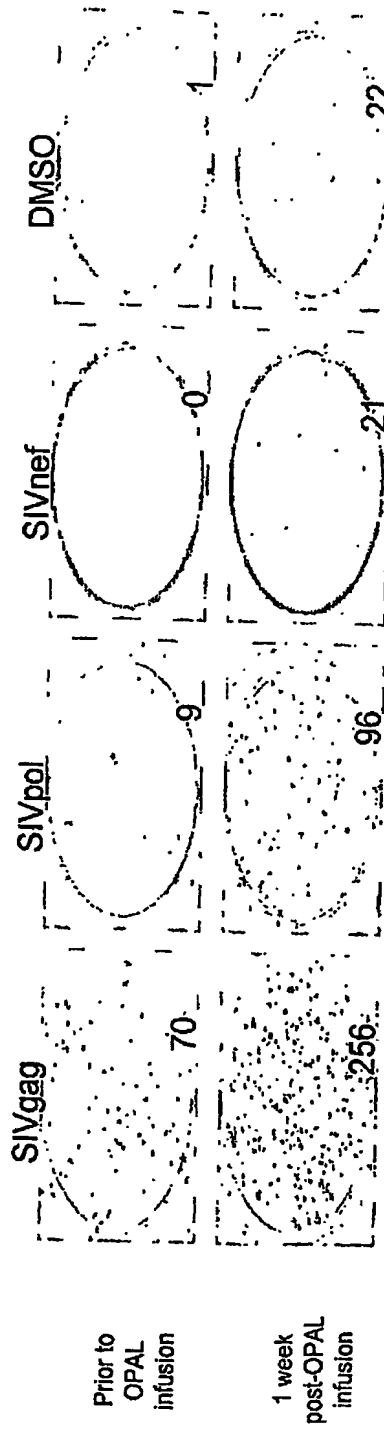


FIGURE 8

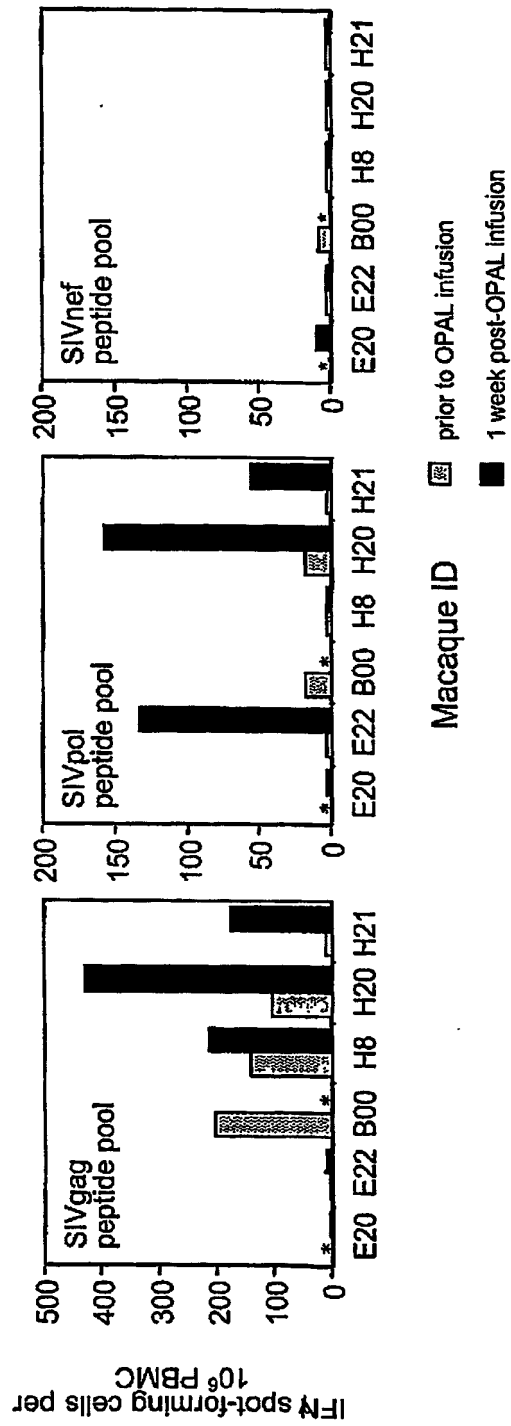


FIGURE 9

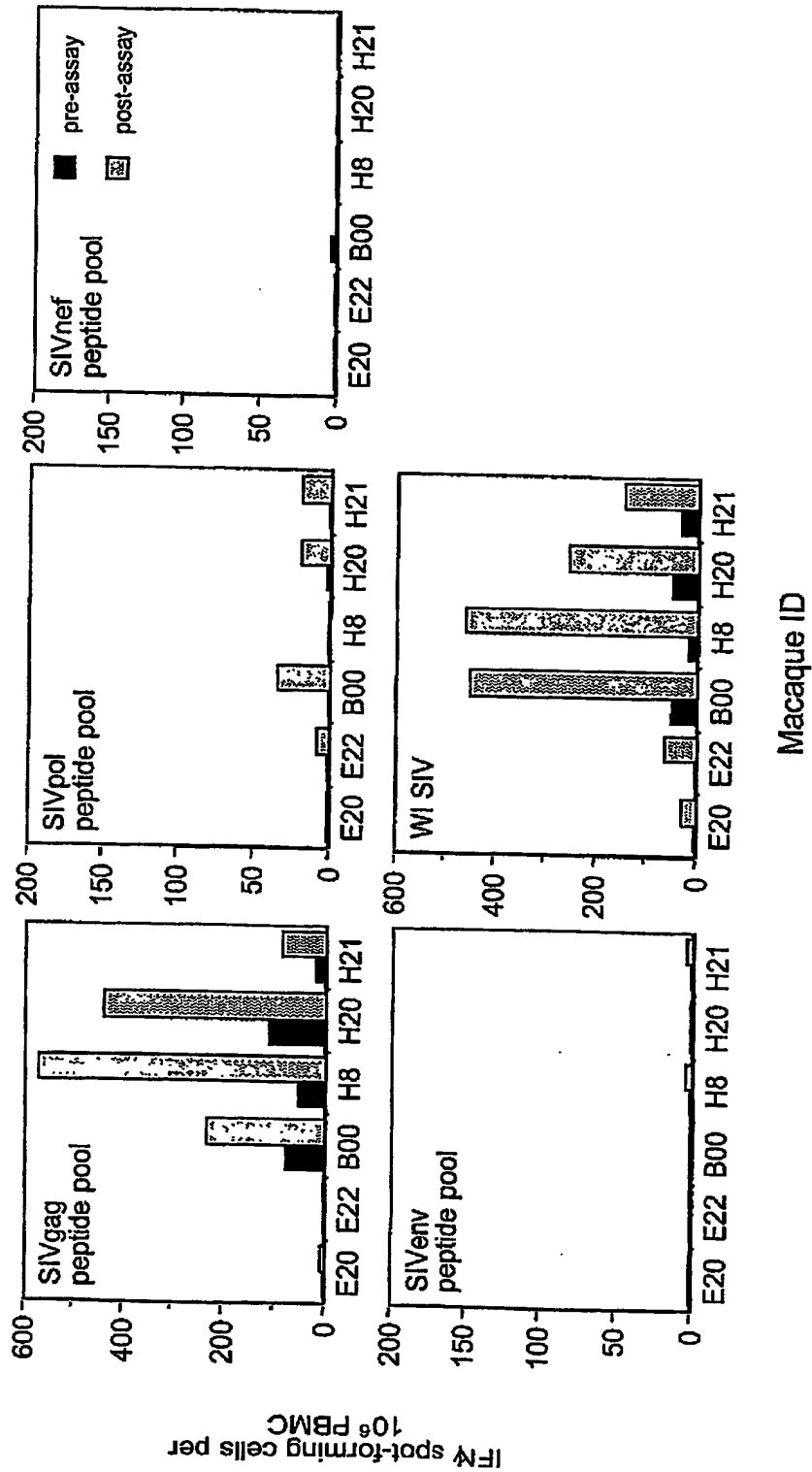


FIGURE 10

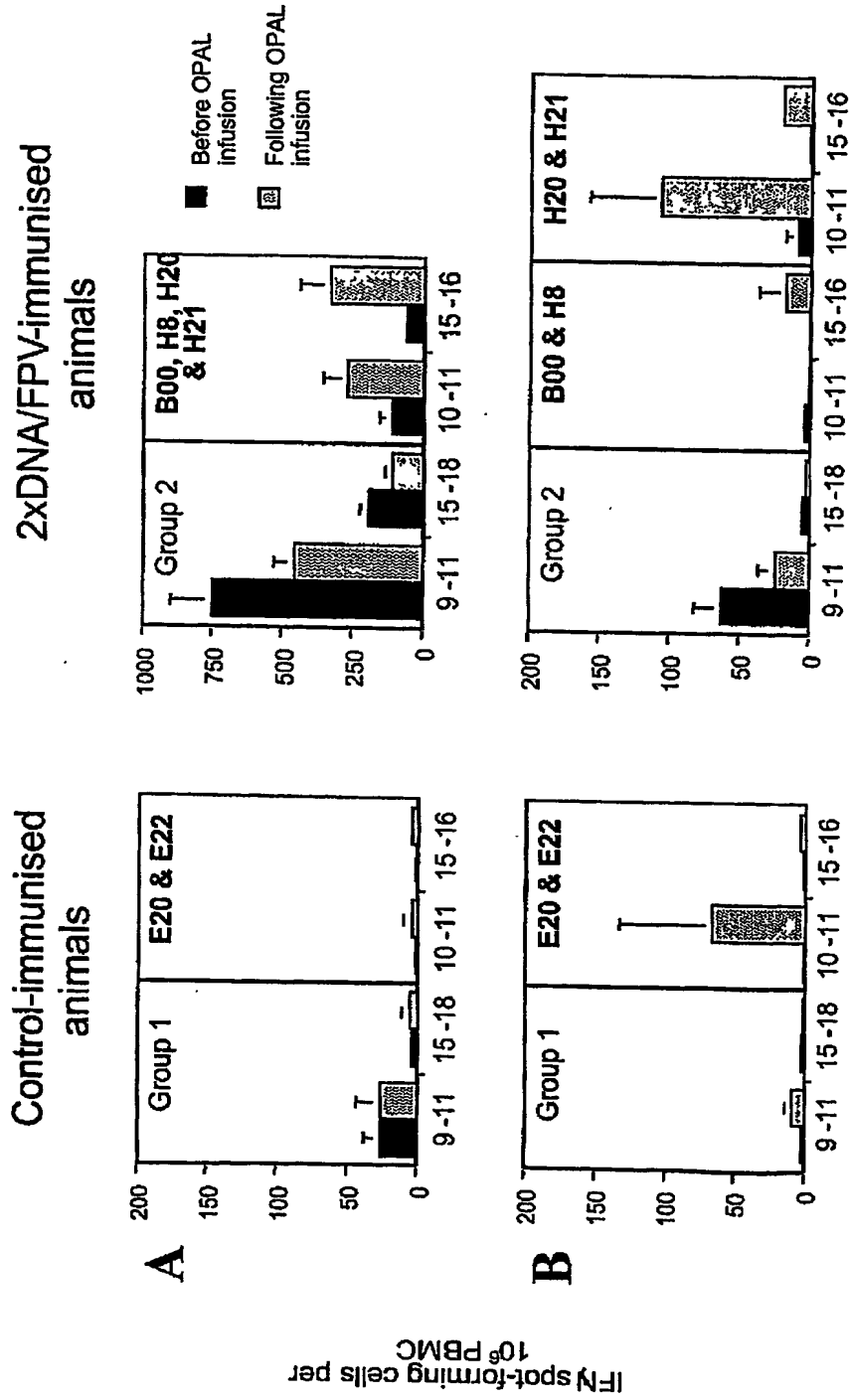


FIGURE 11

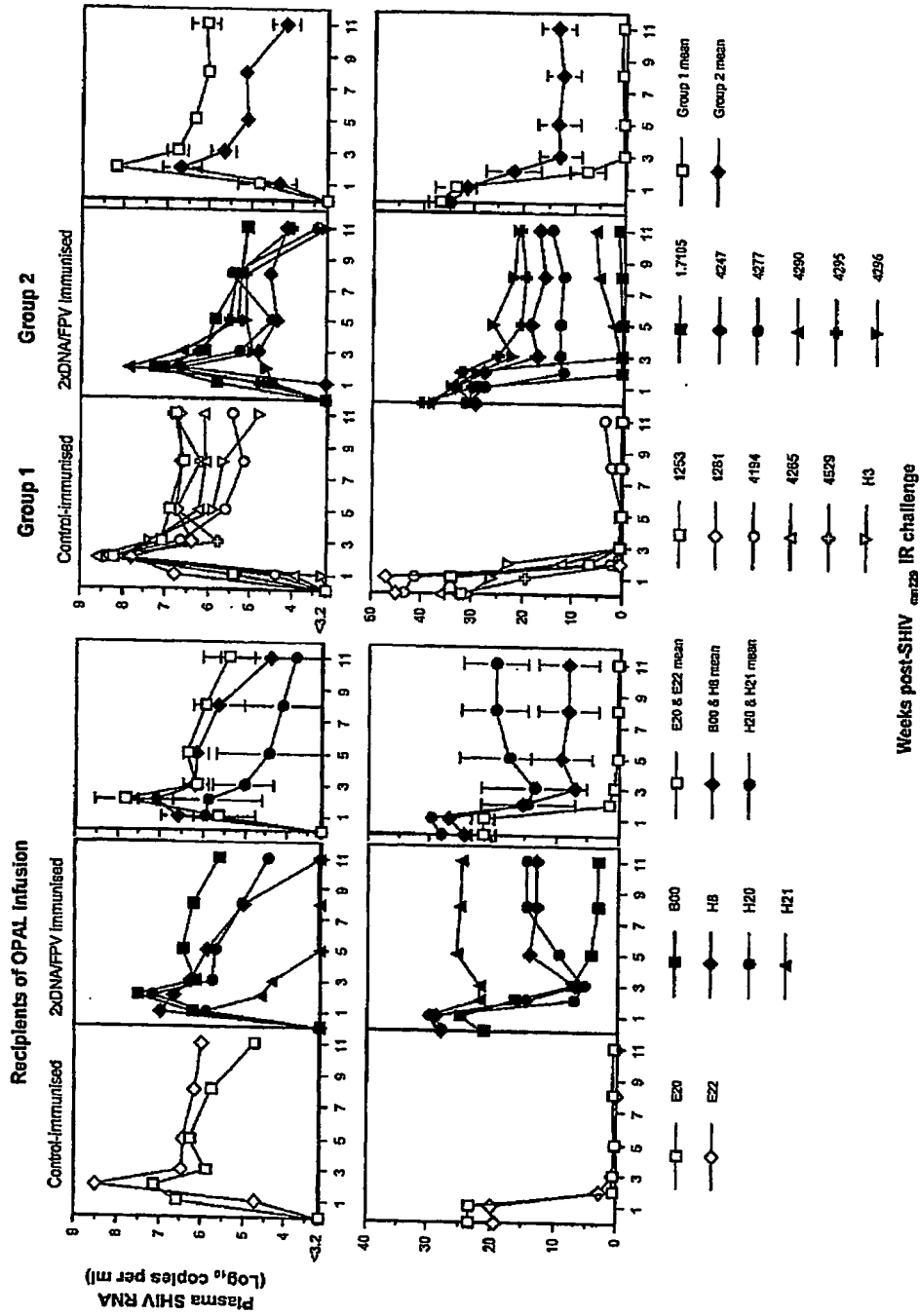


FIGURE 12

MEADASTVSGGELDRWIKIRLPGKKYKXIBIYHASELERFANVKGLETSGCRQ1LQLOP5LQSGSELSHMTVATLYCHORIEVDTKEALKEKIEBQHSKXKQQAADTSSQVSQNTPIVQMLQGMVHQRI5PRTLANVAVVEKAFSPVIP
 (1) KGRASVLSGGELDR
 AVLSGGELDRNEKI
 SGGELDRBKILRP
 LORNEKILRPGCK
 EITLAPGCKKXVZ
 LAPGCKKXVZ
 GUKYKXVZ
 YALXKXVZ
 HVMASRELERP
 (10) ASRELERFANVPG
 ELERFANVPG
 FANVPG
 POLLETSSGCR
 POLLETSSGCR
 ETSGCRQ1LQLOP
 GCRQ1LQLOP
 LQLOP
 LQPSLQSGSELS
 LQPSLQSGSELS
 SSELRLMTVATLY
 (20) ESELRLMTVATLY
 (21) NTVATLYCHORIEV
 TYCHORIEVDTKE
 VORIEVDTKEAL
 TEVOTKEALKEKIE
 DTKEALKEKIEBQ
 ALEKIEBQKXVZ
 IEEQKXVZ
 QKXVZ
 KQQAADTSSQVS
 (30) QQAADTSSQVS
 ADTSSQVSQNTPI
 HSSQVSQNTPI
 VSQNTPIVQMLQGM
 YPIVQMLQGMVHQRI
 QMLQGMVHQRI5PRTLAN
 HQRI5PRTLANVAV
 SPRTLANVAVVEK
 LAMVAVVEKAFSP
 (40) VAVVEKAFSPVIP
 EKA5P5VIMF5SALSGEATPDANTVGLTVGCHQAMQKETTINEBANEVDRHVPAGPIAGQMBREPGSDLAGTSTLQRIQGMVTPPIPVGSIYKSMILGLANKIVMTSPTSILDIRQSPKEPPTVDVRFETLRAQASQSVKMTETILLVQBNPDP
 (41) BEKAP5VIMF5SA
 P5VIMF5SALSGEATPD
 VIMF5SALSGEATPD
 P5SALSGEATPD
 SEGATPDQNTMT
 TPDQNTMTVGH
 LNTVGHQAMQK
 LNTVGHQAMQK
 GCHQAMQK
 (50) ANQKETTINEB
 QKETTINEBANEV
 ETSINEBANEVDR
 BEANEVDRHVPAG
 EMDLHVPAGPIAP
 LHPVAGPIAPQNR
 HAGPIAPQMBREPG
 IAPQMBREPGSDIA
 QMBREPGSDIAGTSTLQ
 (60) DIAGTSTLQSGIGM
 (61) TSTLQSGIGM
 LQSGIGMTPPIPV
 IGMTNPPIPVGH
 THPPIPVGH
 PIPVGH
 GSIYKSMILGLANKI
 KSMILGLANKIVMTSPTS
 ILGLANKIVMTSPTS
 KIVMTSPTSILUI
 (70) ETSPTSILDIRQSP
 PTSILDIRQSPKEP
 LDIRQSPKEPPTVDVRF
 QSPKEPPTVDVRF
 EPPPTVDVRFETLRA
 DYVRFETLRAQASQSV
 RFTILRAQASQSV
 TILRAQASQSVKMTETILLVQBNPDP
 EQASQSVKMTETILLVQBNPDP
 QSVKMTETILLVQBNPDP
 (80) BMTETILLVQBNPDP

FIGURE 13

ETLAVQANPCKTILKALGPAATLEENATACQGGCFQKGRVLAENSVTNSATIMQQRNFQNRATVKGFCNCHGHIANNCPAKGCKHCKGSHQKQCTERQANPLGKINFSHGKPGNVLQSRPEPTAPPEESPRCEHTTTPSQKQEPIDKELYPLA
 (81) ETLAVQANPCKTII
 VQANPCKTILKAL
 NPCKTILKALGPA
 KTLKALGPAATLE
 KALGPAATLEENAT
 PAATLEENATACQV
 LEENATACQGGP
 NTACQGGPCHKAR
 (90) GPCHKARVLAENSV
 KAVLAENSVTNS
 LAENSVTNSATIN
 NSQVNTNSATINQ
 TNSATINQNRQFNI
 TIMQQRNFQNRK
 QRCNFMQRKVC
 FQNRKVCNCG
 RKVACNCGH
 VACNCGHIANN
 (100) NCNCGHIANNCPAP
 AKCNAPKCKCK
 RAFPCKCKCKG
 KCKCKCKGSHQK
 NCNCGSHQKCTE
 KGHQKCTERQAN
 QKQCTERQANFLGK
 CTERQANFLGKIPS
 QNPLGKIPSHKOR
 (110) LGKIPSHKORP
 WPSHKORPGLQSR
 KGRFGNPLQSRPEPT
 GNPLQSRPEPTAPPE
 QSRPEPTAPPEESPR
 EPTAPPEESPRCE
 PPEESPRCEHTT
 SPPEESPRCEHTT
 GSEHTTTPSQKQEP
 TTPSQKQEPIDKEL
 (120) SQKQEPIDKELYPLA

EPIDKELYPLASLSLQNDPSSQ
 (121) EPIDKELYPLASLS
 KELYPLASLSLQND
 PLASLSLQNDPSS
 (124) LKSLQNDPSSQ

FIGURE 13 contd

(11) MGNWNSVGVGHPTVREHRRRAEPADGVGAVSBDLEKHCATTSNTAANWADCALEQBESEVFPFRVQVPLRPPTVLAANDLSHFLKEEGGLEGLITSQKRDQDLDLAWHTQSTFQDNQNTTQGGIRYPLTFCGCFKLVWEPFKVEANDEENNLSLLHPMSLH
NSGNSVGVHPTVREZ
SVYGVHPTVREHRRRA
WPTVREHRRRAEPAA
RESMERAEPAADGVG
ERAEPAADGVGAVSR
PADVGVGAVSBDLEK
GVGAVSBDLEKCAI
VSRDLEKCAITSSN
(10) LEHGRITSSITAA
CATSSITAAANRDC
SSITAAANRDCANLE
AANNAANRDCALQABE
AUCMTEAQBESEVG
WLEAQBESEVGFPR
QBESEVGFVRQVPP
EVGFVRQVPLRPM
PVRQVPLRMYHA
GVPLRMYTQAVOL
(20) RMTTQAVOLSHFL
VSEANDEENNLSLHP
(40) REGENNLSLHPMSLH
EPKVEEENEG
LVPVEKVEEENEG
HCPVLPVPEKVEE
LTGSCPCVLPVPEP
IRYPLTFCPCVLPV
PQIRYPLTFCF
QNTTQGGIRYPLT
FPQNTTQGGIRY
(30) TQSTFQDNQNTTQ
WYHQTGCFPRQY
LDLAWHTQCTFD
KRDLDLAWHTQ
LYSQKRDLDLAWY
LEGILYSQKRDLD
ENGLYSQKRDLD
HFLKAGGLEGLITS
VOLSHPLEKGGLESG
(21) YKAANDLSHFLKENG
MGNWNSVGVGHPTVREHRRRAEPADGVGAVSBDLEKHCATTSNTAANWADCALEQBESEVFPFRVQVPLRPPTVLAANDLSHFLKEEGGLEGLITSQKRDQDLDLAWHTQSTFQDNQNTTQGGIRYPLTFCGCFKLVWEPFKVEANDEENNLSLLHPMSLH

(41) INSLHAPMELHNOODPEREVUWKFPSLSLAFHAPQAREHPEYTCOC
INSLHAPMELHNOOD
LHFWPSLSLAFHNOODPERE
SLHNOODPEREYIWA
NOODPEREVUWKFOS
EREYUWKFOSLSLAF
LHFWPSLSLAFHNO
FOSTRSLAFHNOERU
LAFHAPMAREHPEY
(49) IMAREHPEYTCOC

FIGURE 14

(1) FREDLAFPOQKABEFSESDTRANSPTRELOVWGRDNHLSZSNGADQGVTSFQITLWQRPVITIKIGQIKENLLDTGUDDTVLSESWLPQNNRPKNIGIGSFKVRQYDQILIEICGHAIGTVLVGFTPTVNIIGRILLTQIGCTLNFISPISIEIYVWKLKRP

DUAPFOQKABEFSE
POQKABEFSEQATRA
ABEFSESDTRANSPT
SSEDTTRANSPTREEL
TRANSPTRELOVGR
SPTRRELOVWGRDNH
RELOVWGRDNHLSLSE
VGRDNHLSZSNGAD
(10) DNNLSZSNGADQGVTSF
JSENGADQGVTSFSE
GAURQGVTSFSEFQI
QGVTSFSEFQITLWQ
SFSEFQITLWQRPV
FQITLWQRPVITIKI
LWQRPVITIKIGQOL
WQRPVITIKIGQOL
IKIGQOLKEALLDTG
QOLKEALLDTGANDDT
(20) EALLDTGANDDTVLEE

(21) DTGANDDTVLEEMLP
DDTVLEEMLPQRRKX
LEEWTLFGRHKRPNNI
NLPGRKRPNNIGG2IG
BNKPRMIGG1GGFTIK
KNIGG1GGFIKUROY
GIGGFIKVRQYDQIL
FIKVRQYDQILIEIC
RQYDQILIEICGHNKA
(30) OILFIEICGRGAIGTV
EICGRGAIGTVLVGSP
HMAIGTVLVGFTPTVN
GTVLVGFTPTVNIIGR
VQPTPVNIIGRNNLT
PVNIIGRNNLTQIGC
IGRNNLTQIGCTLNF
LUTQIGCTLNFISP
IGCTLNFISPISIEIYV
LAFPISEIYVVPVKL
(40) ISPIETVPVKLGRM

(41) ETUPYKLPQCGPQKVPQPLTBEKIKALUVEICTEMHEKHSKIGENPNYNTFWPAKKKUSTKRNKLVDFEAKRKQDVEHQVQGIHPBAGIKKKKSVTLVDGQAVSVFVLDQDFKTAFTTFSINNETPGIKQVNLVQGHKSGPAIFQSEWTKILEFFPKQDP
 STVPVLPKQMGQX
 VALKPMQGPQKQK
 PQMGQKVPQKQPLATE
 GPKVQKQPLTEKKIK
 KQPLTEKKIKALIVE
 LTBEKIKALUVEICTE
 KIKALUVEICTEMENS
 LVEICTEMENGLS
 CTMEKSGCKSLGIP
 (50) BEKISKIGENPZY
 KISKIGENPNYV
 IGENPNYVPAIK
 NPYNTPVFAIKKQDS
 TPVFAIKKQDSTKWR
 ATKKQDSTKRLVQ
 KUSTKRLVDFREL
 KXKRLVDFRELHRT
 LVDFRELHRTQDFF
 RELAKTQDFFVQL
 (60) KRTQDFFWEVLGIPH
 (61) DPMVQVQGIHPBAGI
 VQVQGIHPBAGIKKKK
 IHPBAGIKKKKSVTY
 AGLKKKSVTYLWVG
 KMSVTYLWGDQAVF
 VTYLWGDQAVFVPL
 DVGDAIFSVFLQDQF
 AYFSVFLQDQFRAYT
 VFLQDQFRKTAFTI
 (70) KQFRKTAFTIPSIN
 XYTAFTIPSINNETP
 FTIPSINNETPGIAY
 SINNETPGIRVQVNY
 ETPGIRVQVNUVQOC
 IYVQVNUVQVQKGS
 IYVLPQGHKSGPAIF
 PQGHKSGPAIFQSSM
 KGSFPAIFQSSMTKIL
 AIFQSSMTKILEPFR
 (80) SSKTKILEPFRKQNP

FIGURE 15

FIGURE 15 contd

(161) KLELPPKQNFQDVIWQVQDDLYVGSDELICQHQRTKIEELAQHLLANGFTTFDPKQHQGQFFPLMNGVELHPDKNTVOPVLVPEKDSNTVNDIOKLVGKLNASQIYAGIKVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 KLELPPKQNFQDVI
 PFFAQNFQDVIYQNM
 QNPFQIVIVQVQDDLY
 IVIVQVQNDLYVGSDD
 QVPMDDLYVGSDDLEIG
 DLYVGSDDLEIQHRT
 GSDLSIQHQRTKIEEL
 ELQHQRTKIEELQHQ
 HRTKIEELQHQHLLN
 (90) HBLQHQHLLNGFTT
 HQHLLNGFTTFDDK
 LMGFTTFDDKQHQX
 FTFDDKQHQXEPFL
 DQHQEPFLMNGVYELHP
 QHQEPFLMNGVYELHP
 PFLMNGVYELHPDKNT
 HQVYELHPDKNTVOPIL
 HQVYELHPDKNTVOPIL
 LHPDKNTVOPILQHQ
 (100) QVTEVPLTEELAEEL
 KNTVQVTEVPLTEELAEEL
 (101) LPEKDSNTVNDIOKLVGKLNASQIYAGIKVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 DSNVTNDIOKLVGKLNASQIYAGIKVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 VNDIOKLVGKLNASQIYAGIKVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 GCLNASQIYAGIKVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 WASQIYAGIKVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 IYAGIKVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 IYVQKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 QLKQKLGKGTALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 (110) LLEGTFALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 TQALTEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 TEVPLTEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 PUTAEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 EAEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 ELAEELAEELAGNEILKEPVHGCVYTFDSKOLIAQIQGQCGQ
 NREILAGPVHGCVYTFDSKOLIAQIQGQCGQ
 LAGPVHGCVYTFDSKOLIAQIQGQCGQ
 VHGCVYTFDSKOLIAQIQGQCGQ
 YTFDSKOLIAQIQGQCGQ
 (120) SKOLIAQIQGQCGQ

[illegible]

FIGURE 15 contd

(161) LQDSGLEWIVTDSOYALGI IQAQDZSESELVSOI IEQI LKERVYLA WVPABKIGNE QVDELVSAGI RKNVLFQIDKAQEBHRYHSHWRAMASDFHL PPVAKETVASCKQLKEANUGQVDCSPGIMQDCTHLEGKI LVAWVAVASGYIEAVIPARTQDS
 GLEWIVTDSOYALG
 HTVDSOYALGIIQ
 DSQVALGIIQADPK
 ALGIIQADPKSESE
 IQADPKSESELVSO
 PKSESELVSOIEQ
 ESELVSOIEQI LK
 VSOI IEQI LKERVY
 (170) IEQI LKERVYLA W
 IKEKRYLA WPAK
 KYLA WPAKIGG
 ANVPAKIGGNEQV
 AKKIGGNEQVDELV
 IGENEQVDELVSAGI
 EQVDELVSAGI RAVL
 KVSAGI RAVLFG
 AGIRVLFQIDKA
 KVLFGIDQEBH
 (180) LQDSGLEWIVTDSOYALG
 (181) DEACQEBHRYHSHWR
 BEHRYHSHWRAS
 KTHSHWRASDFHL
 NHANASDFHLPPV
 PASDFHLPPVAKET
 PHLPPVAKETVASC
 PVVAKETVASCCKQ
 KEIVASCCKQAGE
 ASCCKQAGEAMIG
 (190) KCQLAGEAMIGQVDC
 KGENHGVDCSPGI
 MUGVDCSPGIMQD
 VDCSPGIMQDCTHL
 PGIMQDCTHLEGKI
 QDCTHLEGKI LVA
 THLEGKI LVA WVA
 GKILLVA WVASGYI
 LVAVVASGYIEAV
 HVASGYIEAVIPAE
 (200) GYIEAVIPARTQDS
 (210) TSTTVACWAGIK
 CHNAGIKQZFIYN
 GLEQZFIYNQSO
 EFGIYNQSOQVIB
 PYPQSOQVIBSNK
 OSQVIBSNKRELK
 VVESNRELKIIQ
 NKRELKIIQVQVQD
 LKIIQVQVQDASHL
 (220) IGVQVQDASHLKTAV
 (221) RQAEHLKTAVQAV
 EHLKTAVQAVFHE
 TAVQAVFHEFKK
 MAYFHEFKKGGIG
 IHNFKKGGIGYSA
 KKKGGIGYSAERI
 GIGYSAERIVOLI
 ISAGERIVOLATI
 ERIVOLATIQTKE
 (230) DILATIQTKELOKQ
 TOIQTKELOKQITKI
 TKELOKQITKIONFE
 QKITKIONFVTRD
 TIKIONFVTRDSRP
 NFRVTRDSRPLANG
 YRDSRPLANGCPAKL
 RDPKANGCPAKLWNG
 WKSPAKLWNGEAV
 AKLWNGEAVIQD
 (240) WKGEAVIQDSOI
 (201) AEVIPARTQDSYAE
 PARTQDSYAEFLZ
 QSTYAEFLZAGRW
 AYFLZAGRWFPVK
 LKLAGRWFPVKITTD
 GRWFPVKITTDGNS
 VNTITDNGSFTST
 HTDNGSFTSTTVA
 GNFSTTVAOWH
 (210) TSTTVACWAGIK
 VQACWAGIKQZFIYN
 CHNAGIKQZFIYN
 GLEQZFIYNQSO
 EFGIYNQSOQVIB
 PYPQSOQVIBSNK
 OSQVIBSNKRELK
 VVESNRELKIIQ
 NKRELKIIQVQVQD
 LKIIQVQVQDASHL
 (220) IGVQVQDASHLKTAV
 (221) RQAEHLKTAVQAV
 EHLKTAVQAVFHE
 TAVQAVFHEFKK
 MAYFHEFKKGGIG
 IHNFKKGGIGYSA
 KKKGGIGYSAERI
 GIGYSAERIVOLI
 ISAGERIVOLATI
 ERIVOLATIQTKE
 (230) DILATIQTKELOKQ
 TOIQTKELOKQITKI
 TKELOKQITKIONFE
 QKITKIONFVTRD
 TIKIONFVTRDSRP
 NFRVTRDSRPLANG
 YRDSRPLANGCPAKL
 RDPKANGCPAKLWNG
 WKSPAKLWNGEAV
 AKLWNGEAVIQD
 (240) WKGEAVIQDSOI

FIGURE 15 contd

GAVVIQNSDIKVPREKAKIIRDYQKMGDCVSRQSD
(241) GAVVIQNSDIKVP
IQNSDIKVPREK
SDIRVPREKAKIIR
VPRGAKIIRDYK
RKAKIIRDYQMG
IIRDYQKMGDCV
YQKMGDCVSRQ
(248) NKEDCVSRQSD

FIGURE 15 contd

FIGURE 16

FIGURE 17

(1) HENH(QVNI)VDQVDEMEIETHKHSYVGHQNTYISRKAKONTYRHYESTHPRISSEVHPLQDARLVITTTTWSGLTCDRWHLCQGVSIERRKIKVSTQVDPDLADQLHLHYFDQVSSSAIRHAILGHIWSPRCEYQAGHNKVSLOTLALAAULITPKIKPPLSPVTLQITE
 HENHQNQVNIHQVDR
 QVNIHQVDRHRIIR
 IYHQVDEMEIETHKHS
 VDEMEIETHKSLVKK
 RIPTFKSLVQVQHMNTI
 WKSILVQVQHMNTI
 WKSILVQVQHMNTI
 VDEMEIETHKSAKGRF
 WYISRKAKGRFYRHH
 BRANQZFYRHHYEST
 (10) CHFYRHHYESTHPRIS
 NHHYESTHPRISSEV
 ESTHPRISSEVHPL
 PRISSEVHPLQDAR
 SEVHPLQDARLVIT
 IPLQDARLVITTTWS
 DARLVITTTKGLTHQ
 VITTTKGLTHQERN
 TNGLTHQERNHILCO
 HTGBRHHQGVSI
 (20) RDNHLLQGVSIERRK

(21) LQGVSIERRKHSYV
 VSIERRKORTSQVD
 WRKKVSTQVDPDLA
 RYSTQVDPDLADQLI
 QVDPDLADQLHLHLYT
 DLADQLHLHYFDQF
 QJLHLHYFDQFSSA
 LXTFDQFSSAIRRA
 OCTFSSAIRRAILGH
 (30) ESAIRRAILGHVSP
 RMAILGHVSPRCEY
 LGHIVSPRCEYQAGH
 VSPRCEYQAGHNKVS
 CEYQAGHNKVSLOY
 AGHNKVSLOYLALA
 KUGLQYALANLJIT
 LQYALANLITPKKI
 ALAANLITPKIKPPL
 LATPKIKPPLSPVT
 (40) KNIKPELSPVTLQITE

FIGURE 18

MEQAPEDQGFQPEPYNENTLLELLESENVRHPTFPAIMHGLAQHIYETVGTAGVEAIIRIQLLFHFRIGQHSRIGITRORRQCSRS
 (1) MEQAPEDQGFQPEPY
 PEDQGFQPEPYNEMT
 GPOPEPYNENTLRL
 EPNENTLLELLEK
 ENTLELLESENVR
 ELLELLESENVRHPT
 ELSENVRHPTFPAIMH
 EAVHPTFPAIMHGLQ
 HEPHPTFPAIMHGLQHIY
 (10) IGLHGLAQHIYETV
 GLQHIYETVGTAGVEA
 HIVETVGTAGVEA
 TYGDTAGVEAIIRI
 TTAGVEAIIRIQLL
 VEAIIRIQLLFIH
 IRIQLLFIHFRIG
 QQLFIHFRIGQHS
 FIHFRIGQHSRIGI
 RIGQHSRIGITROR
 (10) QHSRIGITRORRQ

FIGURE 19

POSIOLATVALVAATIAIIVNSIVFIEFKILQKIDRIDIRERAEOSGSGOEHISALVEMGHAFNDVDDL
 (1) POSIOLATVALVA
 QILALVALVAATIA
 IVALVAATIAIUV
 VVAATIAIIVNSIVF
 IIAIIVNSIVFIEYR
 VMSIVFIEYKILR
 IVFIEYKILRQKI
 EYFIEYKILRQKI
 ILQKIDRIDIR
 (10) RKIDRIDIRERAE
 RLIDIRERAEZDSCH
 RIERERAEZDSCH
 RAEDSCHESGDOEF
 SCHESGDOEF
 SEGDOEFALVENG
 QHELSALVENGHAP
 SALVEMGHAFNDV
 (18) ENGHAFNDVDDL

FIGURE 20

FIGURE 21

[illegible]

FIGURE 21 contd

FIGURE 21 contd

(241) NULGCHLAALAYCSTGVVIGRIVLSCRPALIPDBEVLYQEPDMECSQELPYIQCHMLASQPKQALGLLQIASRQAEVITPAVQTHQKLEVPFAKHQHPISQIQYLAGLSTLPQNPALISLAUTAA
 LAALAYCSTGVV
 AAYCSTGVVIG
 CUSTGVVIGRIVL
 GCWVIGRIVLSGK
 VVIGRIVLSGKPAIPDB
 LSGRPALIPDBEVLYQEP
 IPDBEVLYQEPDMECS
 YQEPDMECSQELPYIE
 (250) EECORLPIVIEQOMLAE
 (251) PTIEQOMLAEQFQNAL
 KLASQPKQALGLLQIAS
 QKALGLLQIASRQAEVIT
 QIASRQAEVITPAVQTHN
 EVITPAVQTHQKLEVPF
 QTHQKLEVPFAKHQHP
 EVFPAKHQHPISQIQYL
 KHPISQIQYLAGLSTLP
 IQYLAGLSTLPQNPALIS
 (260) STLEKPAIASLAUTAA
 ATASLAUTAAVTSPLTTQOTLLFNILGQWVAQAAPQATATFVGLAGLAAIGSVGLGKLVNDILAGYGRAGVAGVAFKINSGRVPTEDLVNLLPAILSPCALWGVVCAILREHVGPQEGJAVQWNRLLAFASRGNHVSPHTVVP
 (261) ATASLAUTAAVTSPLTT
 FTAUTAAVTSPLTTQOTLLFN
 PLTTQOTLLFNILGQWVA
 LFNILGQWVAQAAPQ
 QWVAQAAPQATATFV
 AAPQATATFVGLAGLAA
 AFVGLAGLAAIGSVGLG
 AGVAGLAAIGSVGLGKLVNDIL
 VGLGKLVNDILAGYGRAGY
 (270) VDLAGYGRAGVAGVAF
 (271) GAGVAGVAGVAFKINSGRV
 LVAFKINSGRVPTEDLV
 SCGRVPTEDLVNLLPAIL
 EDLVNLLPAILSPCALW
 PAILSPCALWGVVCAI
 ALWGVVCAILREHVGP
 CAILREHVGPQEGJAVQ
 HWGPQEGJAVQWNRLLAF
 AVQWNRLLAFASRGNHV
 (280) LIPASRGNHVSPHTVVP
 GNVSPHTVVPESDAAREVTAISSLVTVLLARLEQWISSBCTTPCQSGMLRDINDHICEVLSDFKFWLAKLAMPQIPQIPFVQCEYBCTGYNAGGIMHPTCHCGAEITGHEVKNUTPILVGPRTCHNWSGTFPINAYTGTCTPLPAP
 (281) GNVSPHTVVPESDAARE
 HTPVSPESDAAREVTAISSL
 RARVTAISSLVTVLLAR
 LLSLVTVLLARLEQWISS
 TQLLARLEQWISSBCTTP
 HQWISSBCTTPCQSGMLR
 CTPCQSGMLRDINDHICE
 SMLRDINDHICEVLSDFK
 DWICEVLSDFKFWLAKL
 (290) SDFKFWLAKLAMPQIP
 (291) KAKLAMPQIPFVQCEY
 LQCEYBCTGYNAGGIMH
 GYNAGGIMHPTCHCGAE
 IMHPTCHCGAEITGHEV
 GAEITGHEVKNUTPILV
 VKNUTPILVGPRTCHN
 RIVGPRTCHNWSGTFPI
 CHNWSGTFPINAYTGTCT
 (300) TFPINAYTGTCTPLPAP
 ITGPTCTPLPAPKPKARVSAKEVRIWVGDFHVSQNTDNLKPCQIPSPHPTTELDGVLHRAFPCKPLAREVSPFWGLHHVYVQSQLPCEPEPDVAVLTGKLTDSHITAGACRHLARGSPSPHSSASQLSAPSLKATCT
 (301) ITGPTCTPLPAPKPKAR
 LPAPKPKARVSAKEVRI
 FAPKPKARVSAKEVRIW
 AKEVRIWVGDFHVSQNT
 DVSQNTDNLKPCQIPSP
 HVSQNTDNLKPCQIPSP
 YVSQNTDNLKPCQIPSP
 DNLKPCQIPSPHPTTEL
 QIPSPHPTTELDGVLH
 FTELDGVLHRAFPCKP
 (310) RLHRAFPCKPLAREV
 (311) PCWTLREVSFRVLAE
 EVSFRVLAEHYPVQSQL
 GHREYVQSQLPCEPEPD
 GSQIPCEPEPDVAVLT
 PEPDVAVLTGKLTDSHI
 LTGKLTDSHITAGACR
 SHITAGACRHLARGSP
 RHLARGSPSPHSSASQ
 SPHSSASQLSAPSLKATCT
 (320) ASSSASQLSAPSLKATCT

FIGURE 21 contd

[illegible]

FIGURE 21 contd

DDLVVICSGVQEDASLRAFTANTRYSAPOPPPEYDLELITSCSNVSHDGGKVVYTLTRDPTTFLADAMETARHTTQVNSHLENIIMFAPTLHARMILMTHTFFSVLTARDQLEQALNCEIYGACYSIEPLDLP
 (401) DDLVVICSGVQEDAS
 ESAGVQEDASLRAFTEN
 DASLRAFTENRYSA
 FTENRYSAPOPPPP
 YGAPPPPPPOPSYDLELI
 PPQPEYDLELITSCSNV
 LELITSCSNVSHDGA
 SSNSVSHDGGKVVYL
 HCHGKVVYTLTRDPTT
 (410) VYTLTRDPTTLAGAANE
 (411) FTFLADAMETARHTTPV
 ANMETARHTTPVNSHLENI
 HTPVNSHLENIIMFAPTL
 LGRIIMEAPTLHARMILN
 APTLHARMILMTHTFFSVL
 NIMTHTFFSVLTARDQLE
 FSVLTARDQLEQALNCEI
 DQLEQALNCEIYGACYSI
 NCEIYGACYSIEPLD
 (420) YGACYSIEPLDLP
 CYSIEPLDLPPIQLHGLSAPSLHYSYSGEINRVAACLRLKGVPTLRAWHRRASVYRALLSRRRAICCKLFNAVTKLAPLAAAGRLDLSQNTAGYSGODIYHSVSARPPFWFCLLAAAGVGIYLLPNR
 (421) CYSIEPLDLPPIQLHGLS
 DLPPPIQLHGLSAPSLH
 RLHGLSAPSLHYSYSGEIN
 PSLSYSGEINRVAACL
 RAEINRVAACLRLKGVPT
 ANCLRLKGVPTLRAWHRR
 GVPTLRAWHRRASVYR
 WRRASVYRALLSRRRAIC
 VRAELLSRRRAICCKY
 (430) RRRRAICCKYLLPNR
 (431) CHYLENVAVTKLAPL
 WAVTKLAPLAAAGRL
 KUTPAAAGRLDLSQNT
 AGRLDLSQNTAGYSGOD
 GNTAGYSGODIYHSVSH
 SGODIYHSVSARPPFWF
 SVSARPPFWFCLLAA
 RFFNFCCLLAAAGV
 FCLLAAAGVGIYLL
 (440) LLLAAGVGIYLLPNR

FIGURE 21 contd